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13. ABSTRACT ***(Maximum 200 Words)***
Estrogen is a mitogen in most estrogen receptor (ER) positive breast cancers and anti-estrogens like tamoxifen have been the mainline therapies for these types of cancers. Although patients initially respond well to anti-estrogens, resistant tumors often develop within 5-10 years of treatment. The purpose of this research is to develop mechanistically distinct therapeutics by directly blocking the interaction of ER with interacting coactivators required for its transcriptional activity. We have successfully probed the ER:coactivator interaction surfaces using peptides isolated from combinatorial phage display libraries. Several different classes of peptides were identified that recognized specific ligand-bound ER conformations. Using these peptides we were able to determine at least two functionally important protein-protein interaction surfaces on ER. One surface is exposed when the receptor is activated by pure agonists and the other surface is formed only when the receptor is bound by tamoxifen. We found a number of peptides that recognize the agonist-induced conformation which can block estradiol-induced ER transcriptional activity. Additionally, we also identified peptides that recognize tamoxifen-bound ER which can antagonize tamoxifen partial agonist activity when expressed in target cells. Both of these classes of peptides have potential to be developed into peptide antagonists for the treatment of breast cancers.

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FOREWORD

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Chin-yi Chang 07-27-2000

PI - Signature Date

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Manuscripts and Meeting Abstracts

Introduction

The purpose of this research is to develop novel therapeutics for the treatment of breast cancers by targeting estrogen receptor (ER) activity. Estrogen is a mitogen in most ER positive breast cancer cells. More than 60% of ER positive breast tumors respond well to anti-estrogens, such as tamoxifen. Unfortunately, the usefulness of this drug is limited by the ultimate development of resistance within 5-10 years of treatment. Therefore, there is an unmet medical need to develop new drugs for the treatment of primary and tamoxifen-refractory cancers. ER is a ligand-activated transcription factor; the transcriptional activity of this receptor depends not only on the bound-ligand but also on its ability to interact with cellular cofactor proteins. We hypothesized that conformations of ER induced by binding of different ligands serve to recruit different cellular cofactors and consequently determine the receptor activity. We proposed that small peptides, obtained from combinatorial phage display screen using ER as a target, could be used to probe these conformations. In addition, these peptides can also be used to devise peptide-antagonists of ER:coactivator interactions to modulate ER transcriptional activity.

Body

Identification of conformational-sensitive probes for ER. We proposed in the Specific Aim 1, to use phage display technology to identify small peptides that can recognize specific ER-ligand complexes. In collaboration with Novalon Pharmaceuticals and the Duke University Combinatorial Sciences Center, we have screened several random peptide libraries using no hormone, estradiol-activated and tamoxifen-activated ER as targets. Using this approach, we successfully identified several classes of peptides which, when characterized using an *in vitro* ELISA assay, were shown to recognize different receptor conformations when it was activated by different ligands (Figure 1). These peptides can serve as conformational-sensitive probes to detect microallosteric changes within ER, and to fingerprint unique conformations induced by specific classes of ligands. For instance, the αII peptide detects ERα occupied by any ligand. The α/β I class of peptides, however, interact with both ERα and ERβ only in the presence of pure agonists such as 17β-estradiol, diethylstilbesterol (DES), estrone...etc, but not the selective estrogen receptor modulators (SERMs) like tamoxifen, raloxifene and nafoxidine. On the other hand, the α/β V class of peptides recognizes a unique conformational state of ER induced by tamoxifen but not other ligands. These peptides clearly demonstrate that different ligands can induce distinct conformations within ER. We have also obtained several peptides that recognize the conformations of ER when it is complexed to GW7604, a SERM that has different biological activities than tamoxifen. We expect that by comparing the peptide-interacting patterns and the biological activities of different compounds, we might be able to associate certain biological activities of ER with the surfaces exposed when it is bound by specific ligands. Part of this study was conducted after the submission of, but prior to the activation of, this research proposal, and the results were published in the March 1999 issue of PNAS (appendix).

Development of cell based-systems with which to detect ER conformations in vivo. In order to verify that the interactions between these peptides and ER can also occur in a cellular environment, we have developed a cell-based mammalian two-hybrid assay. Briefly, individual peptides are fused to the yeast-Gal4 DNA binding domain, and the interaction of these peptides with a chimeric ER-VP16 fusion protein is measured by assessing the expression of a reporter luciferase gene containing Gal4-response elements. This system allows us to validate the receptor-peptide interactions in a cellular environment and to identify the surfaces on ER exposed *in vivo*. In most cases, the ability of the peptides to recognize ligand-activated ER in the mammalian two-hybrid assays agreed with the data generated *in vitro* (Figure 2). However, several peptides that recognized ER *in vitro*, were not able to interact with ER *in vivo*. This highlights the importance of developing *in vivo* assay systems to validate the *in vitro* binding results. We anticipate that this cell-based system will form the foundation of a high throughput screen, which can be used to identify novel ER ligands with predictable *in vivo* pharmacology. In addition, these cell-based assays have allowed us to cross-screen peptides against different steroid/nuclear receptors that are readily expressed in mammalian cells but difficult to produce in vitro, to screen for receptor specificity of these peptides.

We expect that surfaces exposed in all receptors might represent a binding site for a common cofactor that is shared by most receptors; and surfaces that are unique to ER would imply potential interaction surfaces for ER-specific cofactors. For instance, the α/β I class of peptides interacts with most steroid/nuclear receptors in the presence of their agonists. This class of peptides contains a conserved LXXLL motif (L: leucine, X: any amino acids) that is often

present in the receptor coactivators and is essential for mediating receptor:coactivator interactions(2). On the other hand, the αII-class of peptide seems to be very specific for ERα and does not interact with other receptors tested.

Generating focused libraries and screening for high-affinity peptides. In Specific Aim 2, we proposed to make focused libraries based on consensus sequences obtained from Aim1, in order to select for higher affinity binding peptides. One such library has been made, based on the α/β I class LXXLL motif. High affinity peptides, obtained from this library using estradiol-activated ERα as a target, were further divided into three sub-classes based on their sequence homology (Figure 3). We found that the class I peptides contain a conserved serine at the –2 position and a charged arginine at the –1 position. The class II peptides were grouped together based on a conserved proline at the –2 position and an invariable leucine at the –1 position. Class III peptides contain a serine or a threonine at the –2 and a hydrophobic leucine, isoleucine or valine at the –1 position. Surprisingly, when we compared these LXXLL motifs to the known coactivators, we found that these three classes of LXXLL sequences actually match the LXXLL motifs from three different types of cofactors. Class I resembles the p160 type coactivators such as SRC-1, GRIP-1 and AIB-1(1, 3, 7). The class II LXXLL motifs fall into the TRAP220/DRIP 205-type coactivators that were found to associate with the thyroid hormone and vitamin D receptors *in vivo*(9, 13). With the exception of PGC-1(8), the class III peptides resemble regulators which interact with agonist-bound receptors but inhibit, rather than enhance the transcriptional activity of these receptors. It was originally thought that all the LXXLL motifs are functionally equivalent and permit the coactivator to dock with the nuclear receptors. We found, however, that these three classes of peptides are not only different from each other by their primary sequences, they also interact with ER in different ways.

Not all LXXLL motifs interact with ER in the same manner. Using several ER mutant constructs, we found that the three classes of LXXLL-containing peptides interact differently with ER. We determined that all three classes of LXXLL peptides bind within the coactivator-binding pocket, judging from the loss of binding to the ER-535stop and ER-LL; two mutants that have deletions and mutations that destroy the coactivator-binding pocket (Figure 4). We also observed that the charged residues capping the coactivator-binding pocket seem to be required by the class I, II and p160 type coactivators for interaction(5, 11). These capping residues, however, do not seem to be required by the Class III peptides for interaction, based on the observation that this class of peptides interacts readily with an ER mutant (ER-3x) that has mutations in these charged residues. We suspected that the LXXLL motif and its flanking sequences might contain information dictating how different types of cofactors interact with ER, thus allowing them to carry out different functions. There has been speculation that different classes of coactivators may interact with receptors in a sequential manner. For instance, the p160 type cofactors may bind to the receptors first to modify the chromatin structure, and then the RNA polymerase contacting-DRIP/TRAP class of coactivators bind subsequently to initiate transcription of target genes. We are collaborating with Dr. Fred Schaufele (University of California, San Francisco) to explore this possibility. We co-expressed green fluorescent protein-tagged peptides and blue fluorescent protein-tagged ER to determine the interaction kinetics of these peptides with ER in living cells. Preliminary data suggest that different classes of peptides interact with ER with different kinetics. With these studies we hope to determine

how important residues govern these interactions and to assemble a better picture of how ER transcriptional activity is modulated by different cofactors.

Using LXXLL-containing peptides as ER antagonists. Since the LXXLL peptides resemble the coactivator:receptor binding surface, we next sought to explore the potential of using high affinity LXXLL-containing peptides to disrupt ER:coactivator interaction. Expression of these peptides in the cells, by transient transfection of a plasmid encoding peptide-Gal4DBD fusions, efficiently disrupted ER transcriptional activity. Furthermore, using a peptide, #293, that recognizes estradiol-activated ERβ but not ERα, we were able to specifically disrupt ERβ transcriptional activity with no observable effects on ERα (Figure 5). Since there are no ERα- or ERβ-specific antagonists available right now, this peptide will be very useful in dissecting ERα- and β-mediated activities. More importantly, this result indicated that it is possible to develop *receptor-specific* peptide antagonists by targeting receptor:coactivator interaction surfaces.

Class III-LXXLL peptides may represent an interacting surface of novel coactivators. The observation that class III LXXLL motifs interact with the ER-3x mutant is very interesting for the following reason. ER-3x manifests its transcriptional activity in a very cell type- and promoter-specific manner. In HepG2 cells, ER-3x is as active as wild-type receptor in the presence of estradiol, while having minimal activity in HeLa cells(12). More importantly, overexpression in the cells of class III LXXLL peptides, but not the LXXLL motifs from the known coactivator GRIP-1, lead to the inhibition of ER-3x transcriptional activity (Figure 6). This result indicates that the class III LXXLL peptides might be competing with an endogenous cofactor which binds to ER in a manner different from the GRIP-1 type cofactors, allowing the ER-3x to manifest its activity. None of the coactivators identified so far seems to fit this pattern; thus, we felt a need to identify such cofactors in order to enhance our knowledge of ER signaling. We have therefore performed a yeast two-hybrid screen using the ER-3x hormone-binding domain as bait and identified several clones that interact with both wild-type ER and ER-3x in a hormone-dependent manner. Detailed characterization of these clones is still in progress. Within the next 6-12 months, we hope to be able to define how these other types of ER-interacting cofactors might impact ER pharmacology .

Tamoxifen- and estradiol-induced ER transcriptional activities are mediated by distinct mechanisms. We had previously identified a system that allows tamoxifen to function as a partial agonist. In HepG2 cells using a C3-luc promoter, estradiol functions as a full agonist and tamoxifen exhibits about 1/3 of the agonist activity of estradiol(6). We expected that if the peptides we identified resemble the interaction surfaces between ER and an endogenous coactivator that allows tamoxifen to function as an agonist, then overexpression of this peptide should disrupt this interaction and suppress tamoxifen partial agonist activity. Indeed, over-expression of peptides, α/β III and α/β V, specifically abolished the tamoxifen partial agonist activity, but had no effects on estradiol-induced activity (Figure 7). On the other hand, a peptide in the α/β I class that only recognizes E2-induced conformation had no effect on tamoxifen partial agonist activity. This is a very exciting finding, because it provides a mechanistic explanation for tamoxifen partial agonist activity and also a novel mechanism by which the activity could be inhibited. It is hypothesized that breast cancers become resistant to tamoxifen by clonal expansion of a sub-population of cells that has the ability to recognize tamoxifen as an agonist. We expect that by using peptide antagonists like α/β V or small molecules that mimic

the α/β V structure, it should be possible to circumvent the development of tamoxifen-resistant tumors. We are currently exploring different approaches for introducing peptides into cells to test this hypothesis. In addition to the methods we discussed in the proposal, we are also considering other alternatives that would allow these peptides to be used not only in the cell culture system but also in animal models. One of the approaches we are currently testing is to tag our peptides to a 13-amino acid fragment of HIV-tat protein(4, 10). This approach has been described in the literature and is very effective in delivering peptides and proteins across cell membranes and into different tissues in a whole animal. We have made constructs to produce large quantities of such fusion peptides in bacterial cells. These purified fusion peptides will be tested in a cell culture system to determine the effectiveness of these peptides in inhibiting ER activity in MCF-7 cells which express endogenous ER. If this approach is successful, we will then consider using these peptides in the tamoxifen-resistant tumor model in athymic nude mice.

Key Research Accomplishments:

1. Identified conformational-sensitive probes for ER.
2. Developed a cell-based assay system to probe ER conformations.
3. Identified different classes of LXXLL, coactivator:receptor interacting motifs.
4. Demonstrated that tamoxifen and estradiol-induced transcriptional activities are mediated through different mechanisms.
5. Identified peptide antagonists for estradiol-induced ER transcriptional activity.
6. Identified peptide antagonists that can distinguish between ERα and ERβ.
7. Identified peptide antagonists which block tamoxifen partial agonist activity within intact cells.

Reportable outcomes:

Manuscripts:

1. C. -Y. Chang, J. D. Norris, H. Grøn, L. A. Paige, P. T. Hamilton, D. J. Kenan, D. Fowlkes, D. P. McDonnell
   Dissection of the LXXLL nuclear receptor-co-activator interaction motif using combinatorial peptide libraries: Discovery of peptide antagonists of estrogen receptors α and β. Molecular and Cellular Biology (19): 8226-8239, 1999.

2. J. D. Norris, L. A. Paige, D. J. Christensen, C. -Y. Chang, D. Fan, P. T. Hamilton, D. M. Fowlkes, D. P. McDonnell
   Peptide antagonists of the human estrogen receptor. Science (285): 744-746, 1999.

3. L. A. Paige, D. J. Christensen, H. Grøn, J. D. Norris, E. B. Gottlin, K. M. Padilla, C. -Y. Chang, L. M. Ballas, P. T. Hamilton, D. P. McDonnell, D. M. Fowlkes
   Estrogen receptor modulators each induce distinict conformational changes in ERα and ERβ. Proceedings of the National Academy of Sciences USA (96): 3999-4004, 1999

Abstracts:

1. C. -Y. Chang and D. P. McDonnell
   Identification and characterization of proteins which interact with ER-AF2 in a manner distinct from the p160 class of coactivators. Keystone Symposia: Nuclear Receptors 2000. Steamboat Springs, Colorado. March 25-31, 2000.

2. C. -Y. Chang, J. D. Norris, L. A. Paige, H. Gron, P. T. Hamilton, D. M. Fowlkes and D. P. McDonnell.
   Probing the molecular determinants of receptor-cofactor binding specificity using combinatorial libraries of receptor interacting peptides. American Association for Cancer Research Special Conference---The Steroid Receptor Superfamily, Indian Wells, CA. January, 1999.

Conclusion:

Anti-estrogens play an important role in the treatment of breast cancers. The drug of choice should be able to control the mitogenic activity of estrogen in the breast while preserving its beneficial effects in the bone, cardiovascular and central nervous systems. Peptides obtained from our phage display screen have already been used to demonstrate that SERMs can induce distinct conformational changes within ER. We have also developed a cell-based system to analyze these peptides in order to determine where on the receptor they bind and what will be the impact of blocking these peptide-binding sites on ER pharmacology. As additional peptides are isolated and characterized, we expect to be able to obtain a map of the important surfaces on ER that are required for specific biological activities. This information will be useful in developing and screening for new drugs to use in the treatment of breast cancers. In parallel, we have also confirmed that expression of high-affinity ER-interacting peptides in the cells, by transient transfection, can block ER transcriptional activity. We are currently experimenting with other peptide delivery systems that will enable us to inhibit ER activity in an endogenous setting.

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Figure 1

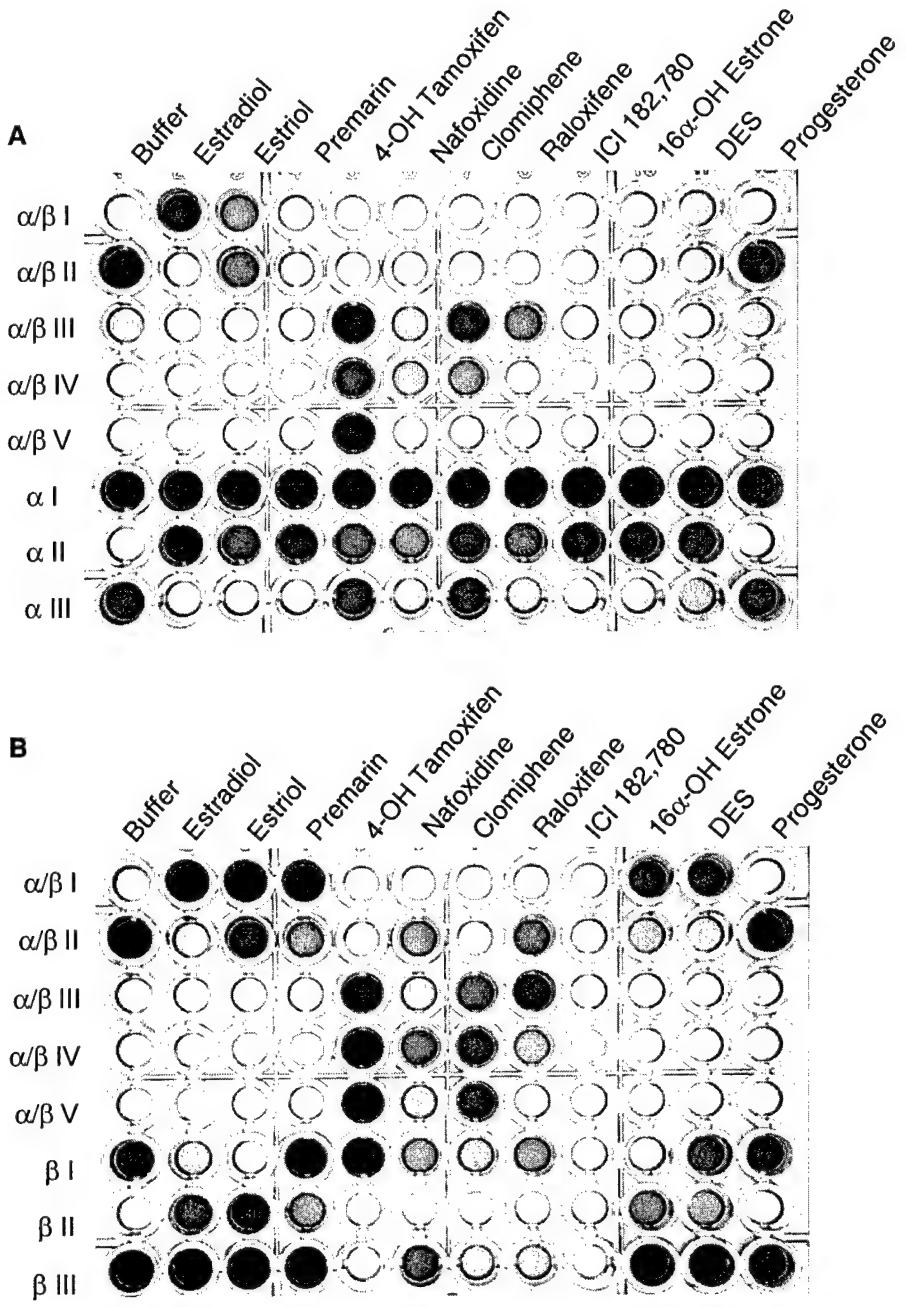


Figure 1. Fingerprint analysis of ER modulators on (A) ERα and (B) ERβ. ERα or ERβ was immobilized on Immulon 4 plate and activated by the different ligands as indicated. Each phage stock was added to the wells and incubated for 30 min at room temperature. Unbound phage were removed by five washes with TBST. Bound phage were detected by using an anti-M13 antibody coupled to horseradish peroxidase. Assays were developed with 2,2-azinobis(3-ethylbenzothiazoline)-6 sulfonic acid and hydrogen peroxide. Absorbance was measured at 405 nm in a Moleular Devices microplate reader.

Figure 2

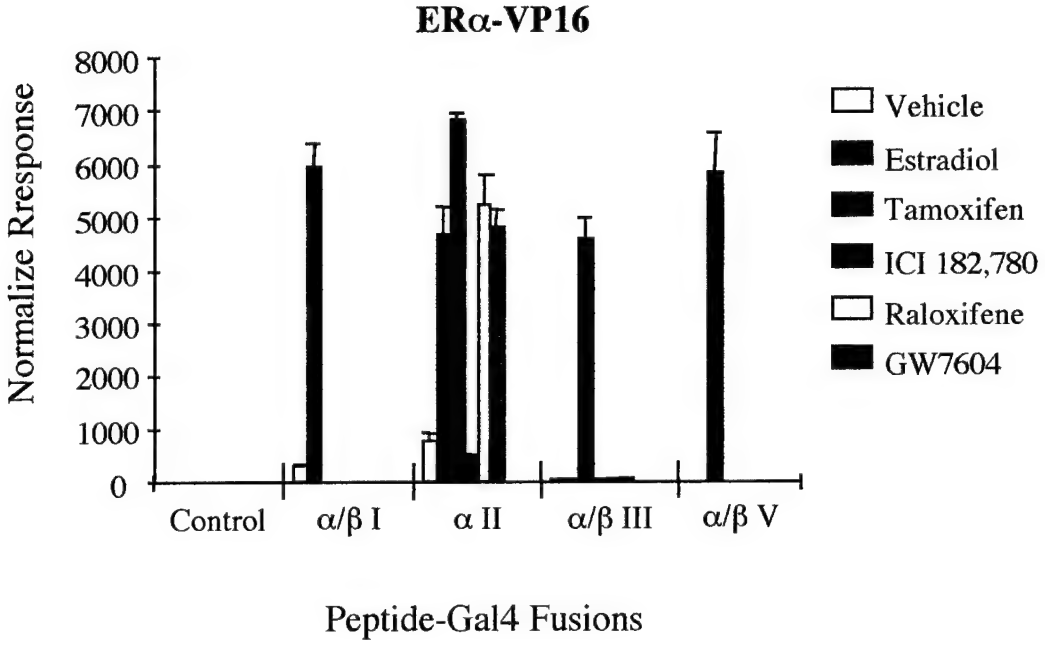
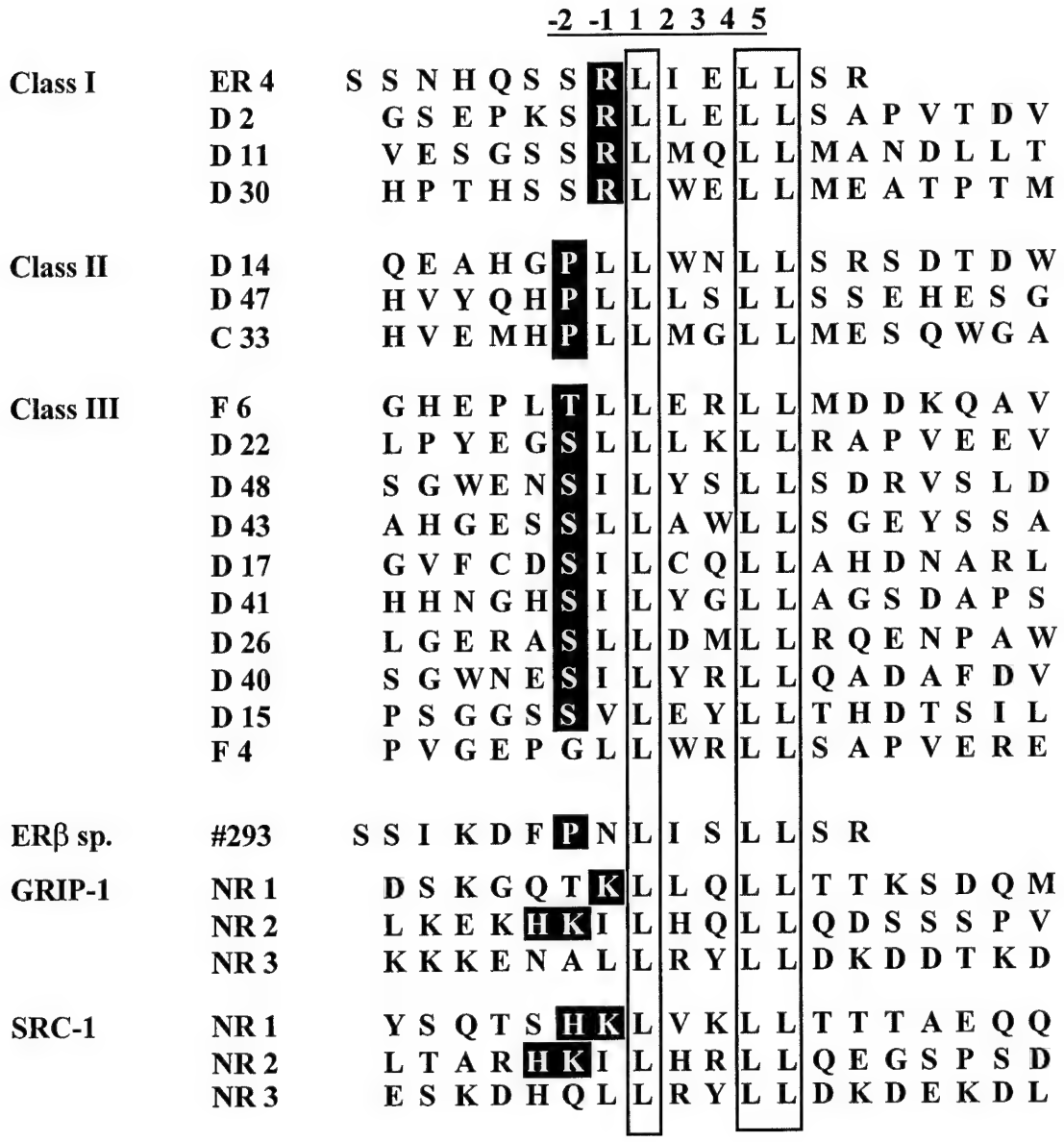


Figure 2. ERα-peptide interactions in mammalian cells. The coding sequence of a peptide representative from each class identified was fused to the DBD of the yeast transcription factor Gal4. HepG2 cells were transiently transfected with expression vectors for ERα-VP16 and the peptide-Gal4 fusion proteins. In addition a luciferase reporter construct under the control of five copies of a Gal4 upstream enhancer element was also transfected along with a pCMV-β-gal vector to normalize for transfection efficiency. Transfection of a Gal4-DBD alone is included as control. Cells were then treated with various ligands (100 nM) as indicated and assayed for luciferase activity.

Figure 3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D 2 G S E P K S R L L E L L S A P V T D V
D 11 V E S G S S R L M Q L L M A N D L L T
D 30 H P T H S S R L W E L L M E A T P T M
Class II
D 14 Q E A H G P L L W N L L S R S D T D W
D 47 H V Y Q H P L L L S L L S S E H E S G
C 33 H V E M H P L L M G L L M E S Q W G A
Class III
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D 22 L P Y E G S L L L K L L R A P V E E V
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SRC-1
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Figure 4

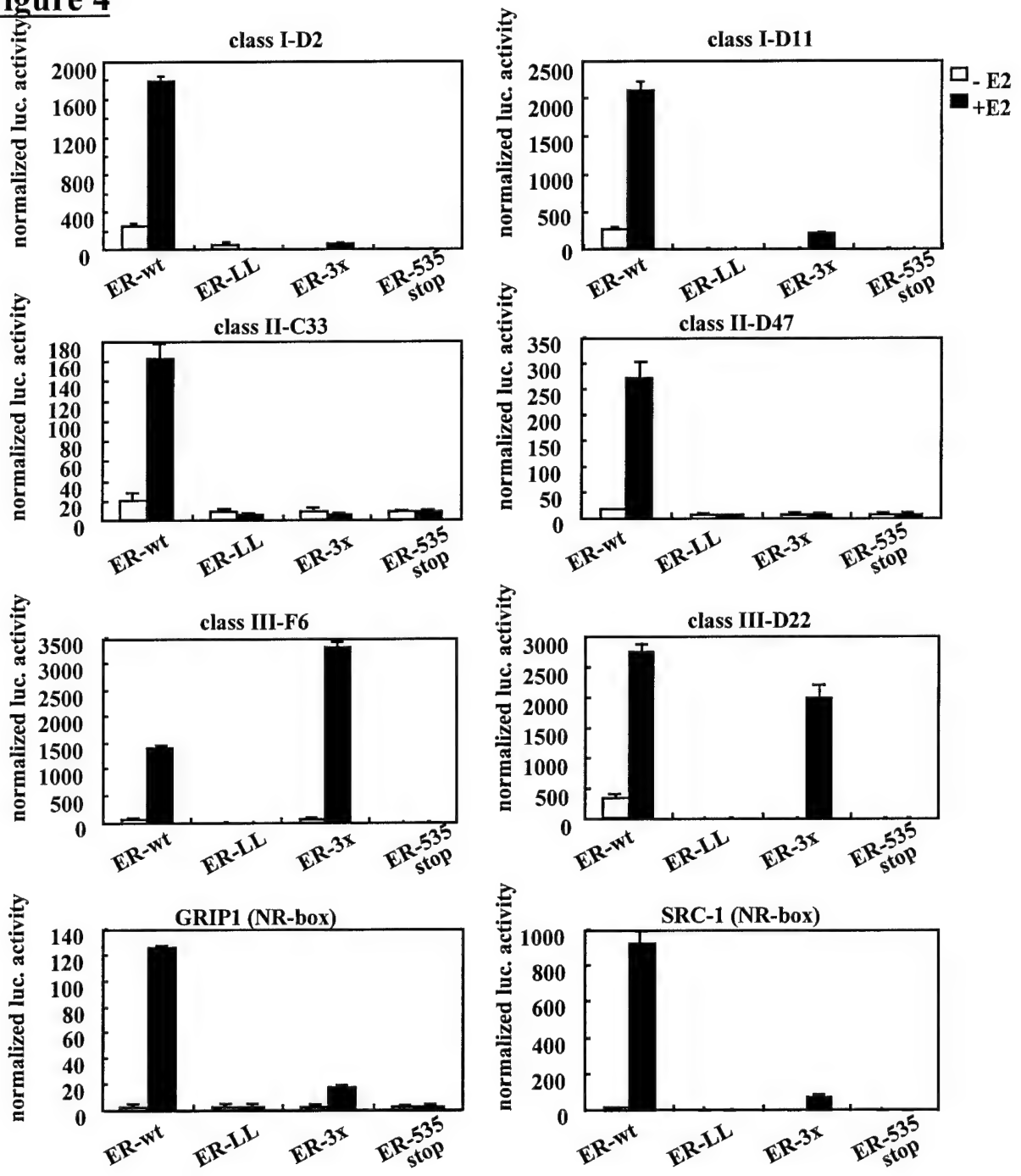

Figure 4. Not all LXXLL peptide-ER interactions require a functional AF-2. Mammalian two-hybrid assays revealed that the three classes of LXXLL-containing peptides interacted differentially with ER helix 12 mutants. Representative clones from each of the three classes of LXXLL-peptides were expressed as Gal4-DBD fusion proteins. Wild-type and mutant ER were expressed as VP16 fusion proteins. The interaction between peptides and ERs was measured by using a 5xGal4Luc3 reporter construct.

Figure 5

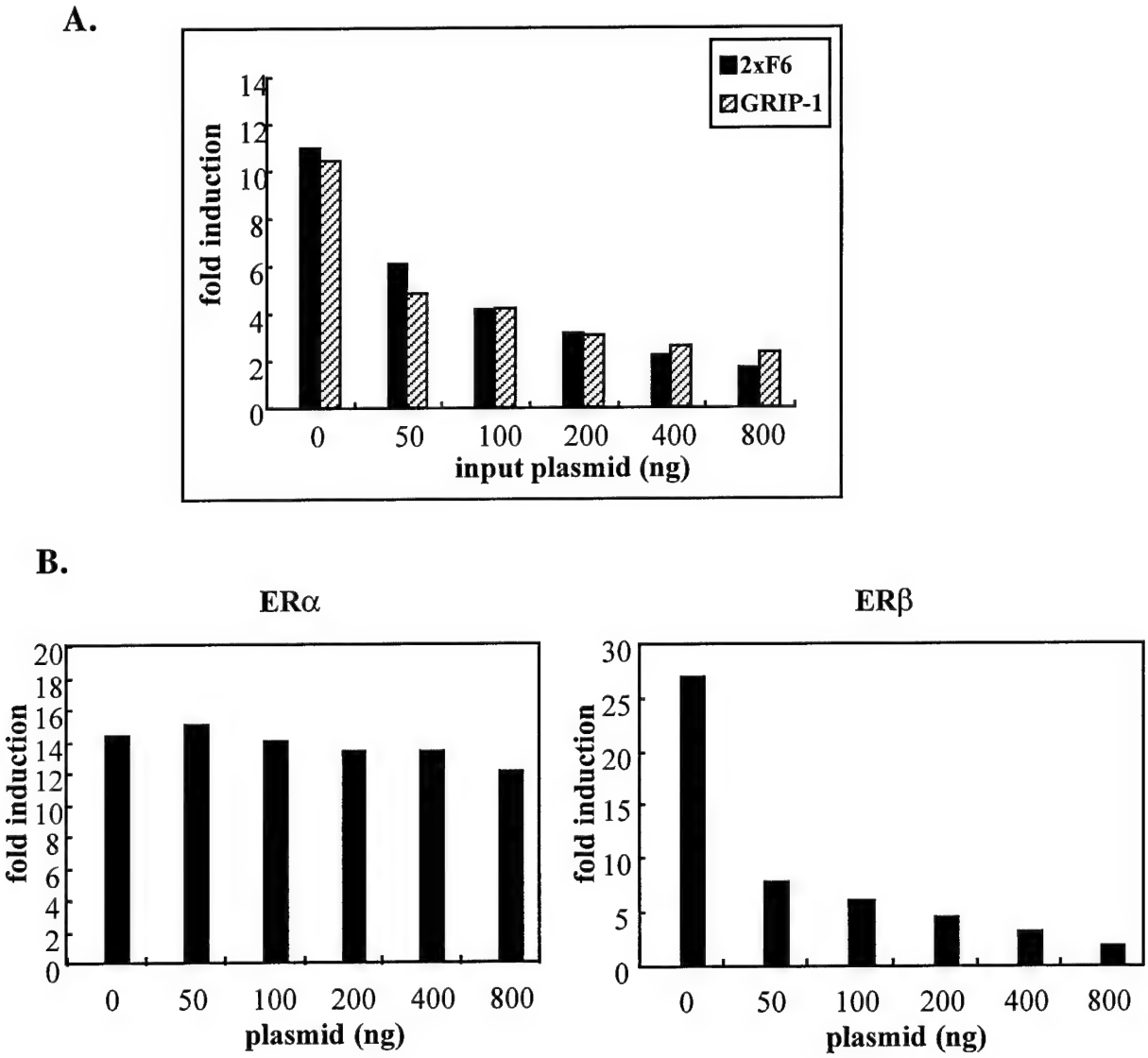

Figure 5. LXXLL-containing peptides disrupted ER transcriptional activity when overexpressed in target cells. (A) HeLa cells were transfected with ERα expression plasmid (RST7-ERα), 3xERE-tata-Luc, along with increasing amounts of plasmids expressing either 2-copy F6 peptide- or GRIP-1 NR box peptide-Gal4DBD fusions as indicated. (B) HeLa cells were transfected with either ERα or ERβ expressing plasmids, 3xERE-tata-Luc, along with increasing amounts of a plasmid expressing ERβ-specific peptide #293. After transfection, cells were induced with 100 nM 17β-estradiol for 16 h before assaying. Fold induction represents the ratio of estradiol-induced activity versus no-hormone control for each transfection.

Figure 6

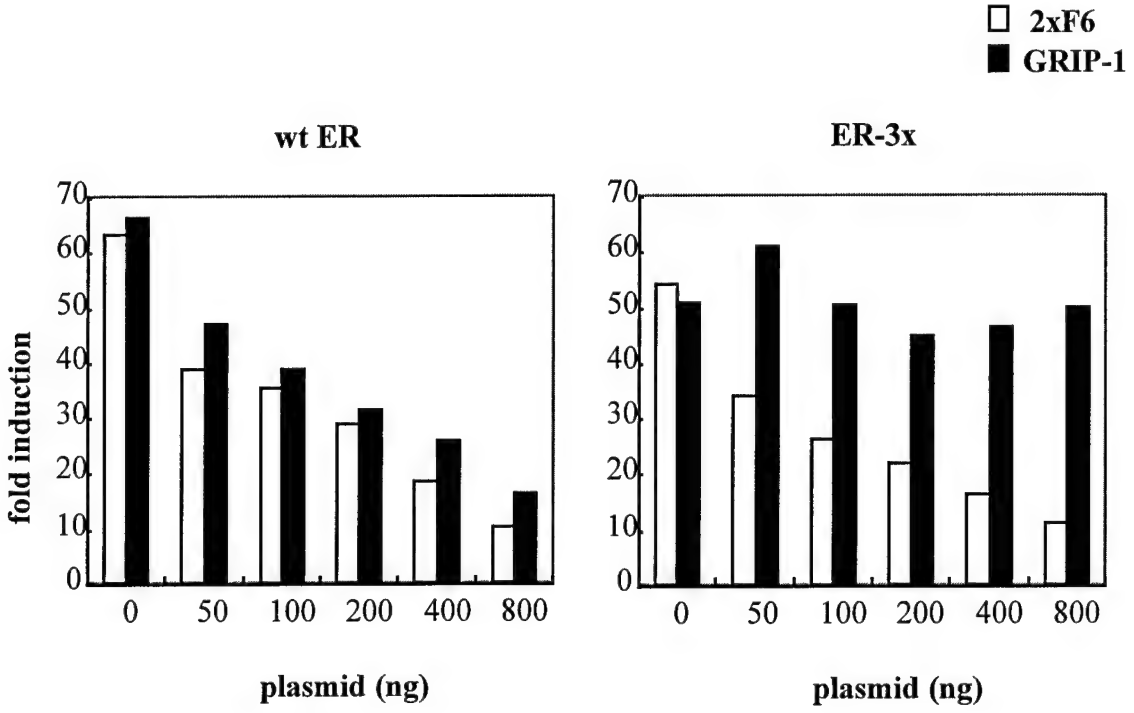

Figure 6. The differential activity of LXXLL-containing peptides to disrupt ERα-mediated transactivation function reveals the presence of multiple ER-interacting coactivators. HepG2 cells were transfected with RST7-ER α (wt) or RST7-ER α-3x mutant expression plasmids along with the 3xERE-tata-Luc reporter gene and increasing amounts of the Gal4-DBD-peptide fusion constructs as indicated. Fold induction represents the ratio of estradiol-induced (100 nM) activity versus no-hormone control for each transfection.

Figure 7

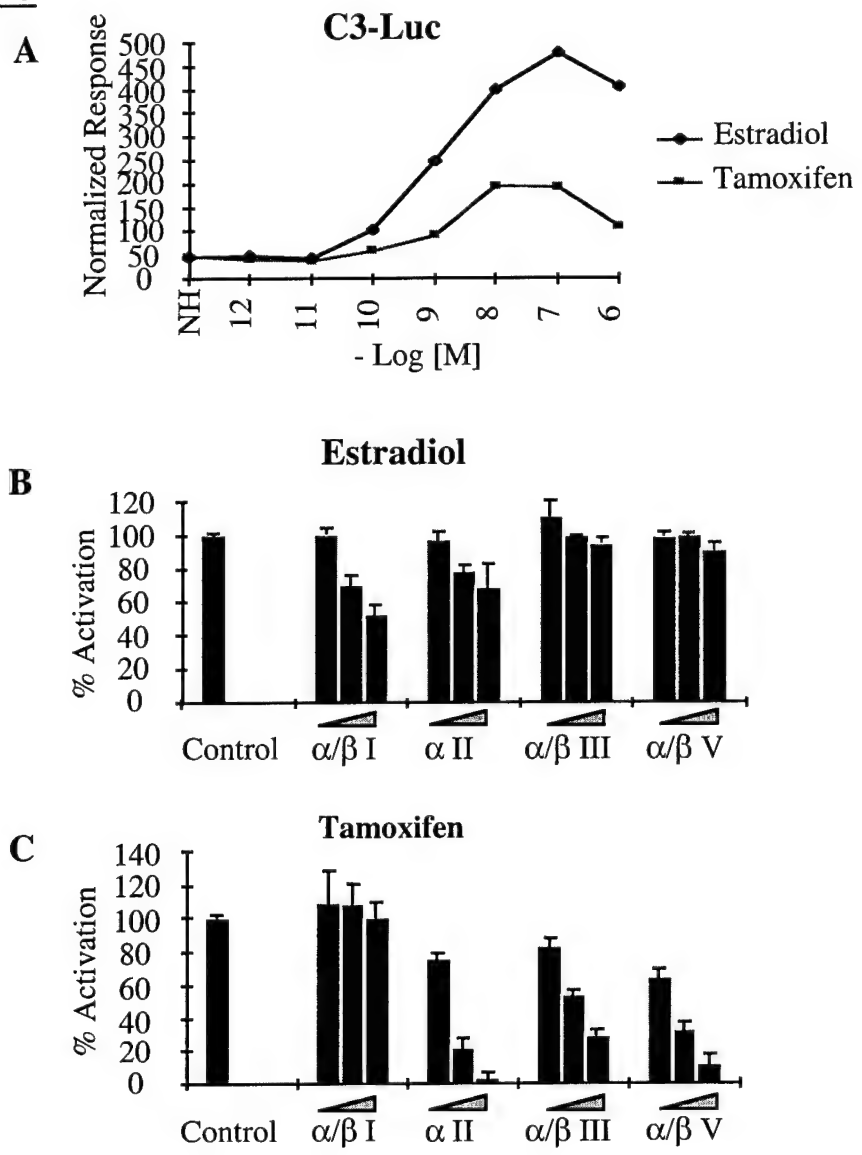

Figure 7. Disruption of ERα-mediated transcriptional activity. (A) HepG2 cells were transfected with the estrogen-responsive C3-Luc reporter gene along with expression vectors for ERα and β-Gal. Cells were induced with either estradiol or tamoxien as indicated and analyzed for luciferase and β-Gal activity. NH-no hormone. (B) HepG2 cells were transfected as in (A) except that expression vectors for peptide-Gal4 fusions were included as indicated. Control represents the transcriptional activity of estradiol (10 nM)-activated ERα in the presence of the Gal4-DBD alone and is set at 100% activity. Increasing amounts of input plasmid for each Gal4-peptide fusion are also shown (◢) with the resulting transcriptional activity presented as percentage of activation of control. (C) Same as in (B) except that 4-OH tamoxifen (10 nM) was used to activate the receptor.

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Dissection of the LXXLL Nuclear Receptor-Coactivator Interaction Motif Using Combinatorial Peptide Libraries: Discovery of Peptide Antagonists of Estrogen Receptors α and β

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Recruitment of transcriptional coactivators following ligand activation is a critical step in nuclear receptor-mediated target gene expression. Upon binding an agonist, the receptor undergoes a conformational change which facilitates the formation of a specific coactivator binding pocket within the carboxyl terminus of the receptor. This permits the α-helical LXXLL motif within some coactivators to interact with the nuclear receptors. Until recently, the LXXLL motif was thought to function solely as a docking module; however, it now appears that sequences flanking the core motif may play a role in determining receptor selectivity. To address this issue, we used a combinatorial phage display approach to evaluate the role of flanking sequences in influencing these interactions. We sampled more than 10^8 variations of the core LXXLL motif with estradiol-activated estrogen receptor alpha (ERα) as a target and found three different classes of peptides. All of these peptides interacted with ERα in an agonist-dependent manner and disrupted ERα-mediated transcriptional activity when introduced into target cells. Using a series of ERα-mutants, we found that these three classes of peptides showed different interaction patterns from each other, suggesting that not all LXXLL motifs are the same and that receptor binding selectivity can be achieved by altering sequences flanking the LXXLL core motif. Most notable in this regard was the discovery of a peptide which, when overexpressed in cells, selectively disrupted ERβ- but not ERα-mediated reporter gene expression. This novel ERβ-specific antagonist may be useful in identifying and characterizing the ERβ-regulated process in estradiol-responsive cells. In conclusion, using a combinatorial approach to define cofactor-receptor interactions, we have clearly been able to demonstrate that not all LXXLL motifs are functionally equivalent, a finding which suggests that it may be possible to target receptor-LXXLL interactions to develop receptor-specific antagonists.

The nuclear receptor superfamily consists of many sequence-related transcription factors that initiate and coordinate the responses to a wide range of physiological signals (13, 24). A simplified model of transcriptional activation by these receptors involves activation of the receptors by their cognate ligands, recruitment of the receptor homo- or heterodimers to target DNA sequences, and subsequent modulation of gene transcription upon interaction with the general transcription machinery. It now appears, however, that nuclear receptor action is more complicated. For instance, most of these receptors are associated with corepressor proteins that silence their activity in the absence of ligands, and activation therefore involves displacement of the associated corepressors by coactivators, an event that permits the functional interaction of the receptor with the cellular transcription machinery (8, 17). Thus, the nature and abundance of these receptor-associated proteins may be a primary determinant of nuclear receptor pharmacology.

A number of coactivators such as SRC-1/NCoA-1 (5, 30), GRIP-1/TIF-2/NCoA2 (16, 48), p/CIP/AIB-1/ACTR (1, 7, 23, 46), and CBP/p300 (9, 12) have been identified and shown to be important for nuclear receptor transactivation. All of these proteins contain a signature LXXLL motif (NR box) which is necessary and sufficient to permit the interaction between receptors and coactivators (15). Results from cocrystallization studies of LXXLL-containing peptides with the ligand-activated hormone binding domains (HBD) of ER and PPARγ demonstrated that these motifs fit into a groove formed by helices 3, 4, 5, and 12 on the receptor (26, 41). Although these structures provided valuable insight into how coactivators dock with steroid hormone receptors, they did not indicate how selectivity of one receptor for a specific LXXLL motif is achieved. It is clear from previous work that each coactivator has specific receptor preferences (11, 15, 19, 25, 49) and that understanding the basis for this selectivity may permit the design of strategies that could be used to target specific receptor-cofactor interactions with novel pharmaceuticals. Preliminary studies, which focused on this problem, have revealed that the two internal residues flanked by leucines within the NR core do not have direct contact with the receptor and do not appear to be important for receptor binding (15, 26, 41). Classical site-directed and alanine-scanning mutagenesis has been used to evaluate how the LXXLL motif interacts with the nuclear receptors and to identify the sequences within the short motif that govern affinity and specificity (11, 15, 19, 25, 49). These studies revealed that sequences N- and C-terminal to the LXXLL motif appear to have the greatest impact on their receptor selectivity and binding affinity (25). However, because of the limited sampling permitted by traditional mu-

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tagenesis approaches, it has not been possible to adequately address the issue of LXXLL specificity and selectivity. For this reason, we have used phage display technology to screen a large combinatorial peptide library in which more than 10^8 combinations of the LXXLL motif was created. This library was then used to probe the nature of the ER-coactivator interaction with a view to identifying the sequences surrounding the LXXLL core motif that are responsible for receptor selectivity and affinity.

Phage display technology has been used successfully in the past to search for peptide sequences that mimic endogenous protein-protein interactions (20, 35, 44). In a previous study, we used this technology to screen for ER-interacting motifs with random peptide libraries and found that LXXLL-containing peptides formed a major sequence cluster when estradiol-activated ER was used as a target (32). Taken together, these data suggested that (i) the information within a short peptide is sufficient to confer specific protein-protein interactions and (ii) the LXXLL motifs appear to be a dominant feature utilized by coactivators to enable them to interact with ligand-activated nuclear receptors. In this study, we further dissected the mechanisms governing the LXXLL motif-ER interactions. Using a phage library enriched for LXXLL-containing peptides to screen for ER interaction sequences, we identified three different subclasses of peptides. All of these peptides interacted with ER in an agonist-dependent manner and mimicked the interaction of coactivators with ER. They differed, however, in their ability to interact with different ER mutants and with other steroid receptors.

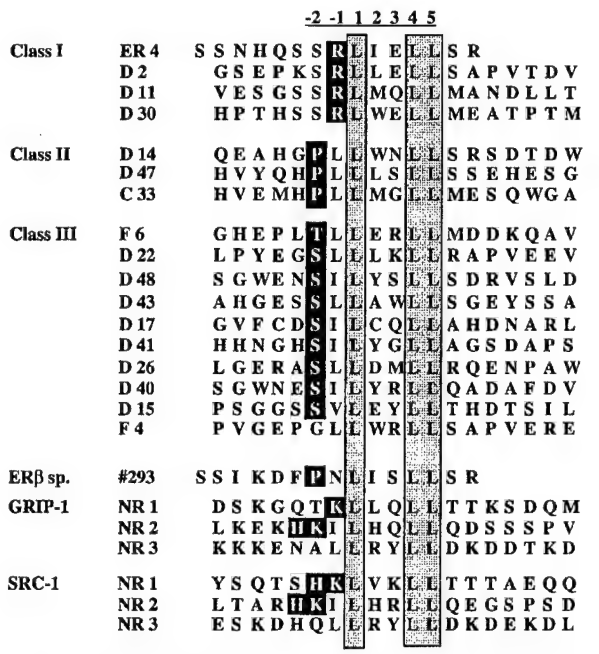


FIG. 1. Affinity selection of ERα binding motifs by using phage display technology. Baculovirus-expressed full-length ERα was treated with 10^{-6} M 17β-estradiol and immobilized on 96-well Immulon 4 plates as a screening target. The LXXLL motif-containing phage peptide library was constructed as described in Materials and Methods. Phage that interacted specifically with estradiol-activated ER were selected, and the peptide sequences were deduced by DNA sequencing. These peptides were classified into three different classes based on sequences flanking the conserved LXXLL motif. Peptide #293 was obtained in a similar manner from random peptide libraries; it bound specifically to estradiol-activated ERβ when analyzed in vitro. Sequences from the center three copies of LXXLL motifs in the SRC-1 and GRIP-1 coactivators are also included for comparison. For reference, we have defined the first conserved leucine as position 1.

MATERIALS AND METHODS

Abbreviations. ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; GR, glucocorticoid receptor; PR-A and PR-B, progesterone receptor isoforms A and B; AR, androgen receptor; TRβ, thyroid hormone receptor beta; RARα, retinoic acid receptor alpha; RXRα, retinoid X receptor alpha; VDR, 1,25-$(OH)_2$-vitamin D_3 receptor; PPARγ, peroxisome proliferator-activated receptor γ; GRIP-1, glucocorticoid receptor interacting protein 1; SRC-1, steroid receptor coactivator 1; RIP140, receptor-interacting protein 140; TRAP220, the 220-kDa TR-associated protein; DAX-1, dose-sensitive sex reversal-AHC critical region on the X chromosome gene 1; SHP, short heterodimer partner; PGC-1, PPARγ coactivator 1; HBD, hormone binding domain; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Gal4DBD, Gal4 DNA binding domain; SERM, selective estrogen receptor modulator.

Chemicals. 17β-estradiol, 4-hydroxytamoxifen, 9-*cis*-retinoic acid, dexamethasone, diethylstilbesterol, 5α-dihydrotestosterone, T3 (3,3′,5-triiodo-L-thyronine), and progesterone were obtained from Sigma Chemical Co. (St. Louis, Mo.); Δ8,9-dehydroestrone, equilin, and estrone were kindly provided by M. Dey (Wyeth-Ayerst Pharmaceuticals, Radnor, Pa.); ICI 182,780 was a gift from A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, United Kingdom); GW7604 was provided by T. Willson (Glaxo Wellcome Research and Development, Research Triangle Park, N.C.); and 1,25-dihydroxyvitamin D_3 was purchased from Duphar Pharmaceuticals (Daweesp, The Netherlands).

Cell culture and transient transfection. Human cervical cancer (HeLa) and hepatoma (HepG2) cells were cultured in minimum essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Life Technologies, Inc.) and maintained in a humidified 37°C incubator with 5% CO_2. For transient transfections, cells were split into 24-well plates 24 h before transfection. Lipofectin (Life Technologies, Inc.)-mediated transfection has been described in detail previously (27). A DNA-Lipofectin mixture containing a total of 3,000 ng of plasmid in each of triplicate samples was incubated with cells for 3 to 5 h, and transfection was stopped by replacing the transfection mix with fresh medium (minimal essential medium without phenol red) containing 10% charcoal-stripped serum. Receptor ligands were added to the cells 14 to 16 h before the assay. Luciferase and β-galactosidase activities were measured as described (27). In mammalian two-hybrid assays, for a typical triplicate of transfection, 2,000 ng of 5×Gal4Luc3 reporter plasmid, 400 ng of receptor-VP16 fusion, 400 ng of pM (Gal4DBD)-peptide fusion constructs, and 200 ng of normalization plasmid pCMVβgal were used. For ER transcription disruption assays, 1,600 ng of 3×ERE-TATA-Luc reporter, 200 ng of pCMVβgal, 400 ng of either pRST7ERα, pRST7ERβ, or other receptor mutant constructs, and 0 to 800 ng of pM-peptide fusion plasmids were used as indicated in the figure legend. The parent pM vector (Gal4DBD without peptide fusion) was used in these experiments to balance the amount of input DNA in transfections. All transfections were performed at least three times; data shown are results of representative experiments.

Construction of the phage library. A focused peptide library in the format of $(X)_7$LXXLL$(X)_7$, where X is any amino acid and L is leucine, was constructed essentially as described previously with the M13 phage-based cloning vector mBAX (43). The top-strand oligonucleotide 5′-AGTGTGTGCCTCGAGA $(NNK)_7$CTG$(NNK)_2$CTGCTG$(NNK)_7$TCTAGACTGTGCAGT-3′ (N = A, C, G, or T; K = C or T) was purchased from Life Technologies, gel purified, and annealed to its complementary-strand oligonucleotide, 5′-ACTGCACAGTCTA GA-3′. The resulting DNA complex was extended with Klenow polymerase in the presence of deoxynucleoside triphosphates to generate double-stranded DNA and was subsequently digested with *Xho*I and *Xba*I and ligated into the mBAX vector, previously digested with the same restriction enzymes. The ligated products were electroporated into *Escherichia coli* JS-5 cells and amplified on 2YT (Life Technologies, Inc.) plates for 6 h to create the $(X)_7$LXXLL$(X)_7$ peptide library. The amplified phage were then eluted from the plates with PBS, concentrated, and finally resuspended in 20% glycerol–PBS and stored at −70°C in 500-μl aliquots. The library has a complexity of 1.5×10^8 different peptide sequences.

Affinity selection of ERα-binding sequences. Baculovirus-expressed full-length ERα was provided by PanVera Corp. (Madison, Wis.). Approximately 0.25 μg (4 pmol) of ERα was diluted in 100 μl of $NaHCO_3$ (pH 8.5) plus 10^{-6} M 17β-estradiol, applied to a single well in a 96-well Immulon 4 plate (Dynex Technologies, Inc.), and incubated at room temperature for 3 h. An equal amount of BSA was added to the adjacent well as a control target. The wells were blocked with 150 μl of 0.1% BSA in $NaHCO_3$ for an additional 1 h at room temperature and washed five times with PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 [pH 7.3], 0.1% Tween 20) to remove excess protein. Then 25 μl of the phage peptide library (with $>10^{10}$ phage particles) diluted in 125 μl of PBST with 10^{-6} M 17β-estradiol and 0.1% BSA was added to the wells, and the plate was sealed and incubated for 8 h at room temperature. Nonbinding phage were removed by washing the wells five times with PBST. The bound

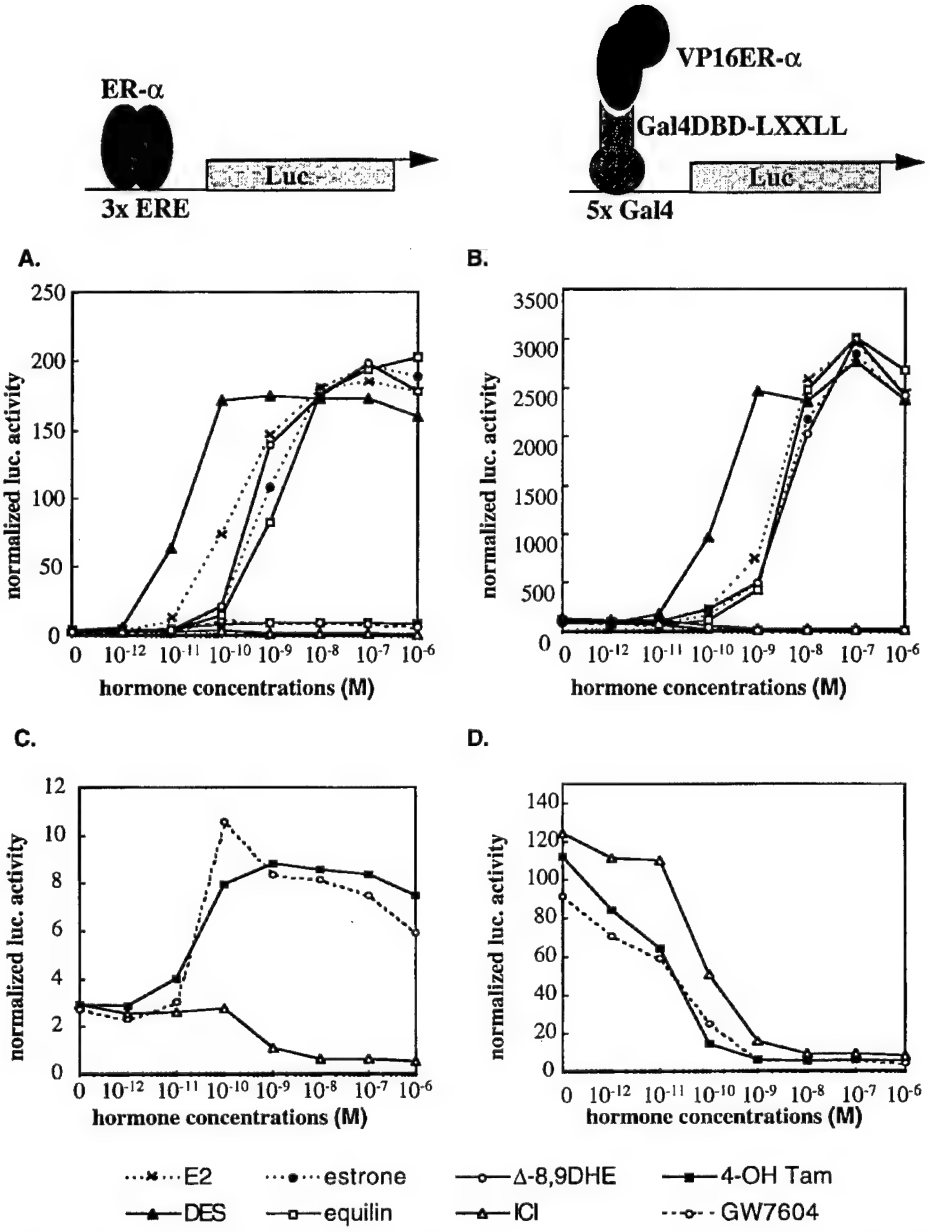


FIG. 2. The interaction between LXXLL-containing peptides and ER occurs only in the presence of receptor agonists. The LXXLL-containing ER4 peptide sequence was fused to Gal4DBD, while the full-length ERα was expressed as a VP16 transactivation domain fusion protein. The interaction between ER4 peptide and ERα was assessed by using the 5×Gal4Luc3 reporter gene (B and D). The ability of different ER ligands to facilitate LXXLL peptide-ERα interactions was compared to the ability of these ligands to induce ER-mediated transactivation, as assayed by using the 3×ERE-TATA-Luc reporter (A and C). HepG2 cells were transiently transfected with the ERα expression vector (pRST7ERα) and its reporter 3×ERE-TATA-Luc construct (A and C) or Gal4DBD-ER4, pVP16-ERα, and 5×Gal4Luc3 (B and D) and treated with different ER ligands as indicated in the key. Luciferase (Luc) activity was normalized to the activity of the cotransfected pCMVβgal plasmid. E2, 17β-estradiol; 4-OH Tam, 4-hydroxytamoxifen; ICI, ICI 182,780; DES, diethylstilbesterol; Δ-8,9DHE, delta-8,9-dehydroestrone.

phage were eluted with 100 μl of prewarmed (50°C) 50 mM glycine-HCl (pH 2.0) followed by 100 μl of 100 mM ethanolamine (pH 11.0). The first eluent was neutralized by adding 200 μl of 200 mM Na_2HPO_4 (pH 8.5) and combined with the second eluent. Phage eluted from the targets were amplified in *E. coli* DH5αF′ cells for 8 h, and the supernatant containing amplified phage was collected for use in subsequent rounds of panning. A total of three rounds of panning were performed. Enrichment of ER binding phage was confirmed by enzyme-linked immunosorbent assay as described below. Individual phage were plaque purified after the third panning, and the peptide sequences were deduced by DNA sequencing.

Enzyme-linked immunosorbent assay. Full-length ERα (0.4 pmol per well) was activated by different ER ligands and coated on 96-well Immulon 4 plates as described above. Then 50 μl of phage stock was applied to the wells and incubated with the targets for 1 h at room temperature. Unbound phage were removed by five washes with PBST. A 1:5,000 dilution of horseradish peroxidase-conjugated anti-M13 antibody (Amersham)–PBST was added to the wells, and

the mixture was incubated for 1 h at room temperature and then washed five times with PBST. Bound antibody-enzyme conjugate was detected by ABTS (2′,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) in the presence of 0.05% H_2O_2, and the color change was measured at 405 nm on a plate reader (Multiskan MS; Labsystems).

Plasmids. All the Gal4DBD-peptide fusions were constructed as follows. DNA sequences coding for the peptides were excised from mBAX vector with *Xho*I and *Xba*I and subcloned into the pMsx vector (derived from the pM vector [Clontech] with a linker sequence to generate in-frame *Sal*I and *Xba*I sites for cloning). The fusion constructs expressing two copies of the LXXLL motifs, 2×F6 and 2×293, were derived from their corresponding single-copy peptide-DBD fusion plasmids by adding a linker sequence (adapted from the sequences found between the GRIP-1 NR box 2 and box 3). Subsequently, a second copy of the LXXLL peptide was added, resulting in the two copies of LXXLL motifs being separated by 50 amino acids, the same spacing found between the GRIP-1 NR box 2 and box 3. The pVP16ERα construct was generated by PCR of the full-length human ERα cDNA with primers containing *Eco*RI sites flanking both 5′ and 3′ ends, and the resulting PCR product was subcloned into the *Eco*RI site in the pVP16 vector (Clontech). pVP16ERβ, pVP16RARα, and pVP16RXRα were generated in a similar fashion. pVP16VDR was a gift of J. W. Pike (University of Cincinnati, Cincinnati, Ohio); VP16TRβ expression plasmid (pCMX-VP-F-hTRβ) was provided by D. D. Moore (Baylor College of Medicine, Houston, Tex.); and VP16GR, VP16PR-A, VP16PR-B, and VP16AR expression plasmids were gifts from J. Miner (VP16GR), D. X. Wen (VP16PR-A and VP16PR-B), and K. Marschke (VP16AR) (Ligand Pharmaceuticals, San Diego, Calif.). Plasmids expressing VP16-ERα mutants were constructed by excision of mutant ER cDNAs from their corresponding expression plasmids (ER-TAF1 [ERα-3×], ERα-LL, and ERα-535 stop plasmids [28, 47]) and subcloned into the pVP16 vector. The VP16-ERα point mutants (ER-D538N, ER-E542Q, and ER-D545N) were generated by using the QuikChange site-directed mutagenesis kit (Stratagene) with wild type pVP16-ERα as a template. Mammalian expression plasmids for ERα, ERβ, and ER179C, as well as the 3×ERE-TATA-Luc reporter construct, are described elsewhere (47). The 5×Gal4Luc3 plasmid was modified from 5×Gal4-TATA-Luc (a gift from X. F. Wang, Duke University, Durham, N.C.) by replacing the luciferase gene with a modified version of luciferase cDNA from the pGL3 basic vector (Promega). GRIP-1 (NR-box) and SRC-1 (NR-box) constructs were generated by subcloning PCR products corresponding to GRIP-1 amino acids 629 to 760 and SRC-1 amino acids 621 to 765 into the pM vector (13a). All PCR products were sequenced to ensure the fidelity of the resultant constructs. An expression plasmid for TRAP220 (pCIN4-TRAP220) was provided by R. Roeder (Rockefeller University, New York, N.Y.). Full-length GRIP-1 and RIP140 expression plasmids were made in the pcDNA3 vector (Invitrogen) by ligating full-length GRIP-1 and RIP140 cDNAs excised from pGRIP1/fl (provided by M. Stallcup, University of Southern California, Los Angeles, Calif.) and pEF-RIP140 (provided by M. Parker, Imperial Cancer Research Fund, London, United Kingdom), respectively.

Western blot analysis. Western blotting was performed with nuclear extracts isolated from HeLa cells transfected with each of the Gal4DBD-peptide fusion plasmids together with a green fluorescent protein expression vector (pEGFP-C3) for normalization purposes. Nuclear extracts were prepared as described previously (38). A 20-μg portion of protein from each extract was separated on an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories). The blots were first probed with an anti-Gal4DBD monoclonal antibody (Santa Cruz Biotechnology, Inc.) to detect peptide fusions and subsequently probed with an anti-green fluorescent protein polyclonal antibody (Clontech) to detect the coexpressed EGFP. The immunocomplexes were visualized by enhanced chemiluminescence (Amersham Corp.) as specified by manufacturer.

Receptor-cofactor in vitro pulldown assays. A 4-pmol quantity of baculovirus-expressed full-length ERα or ERβ (each obtained from Panvera) was immobilized on Immulon 4 plates and blocked as described above. An equal amount of BSA was added to the adjacent wells as a "no-receptor" control target. ^{35}S-labeled RIP140, GRIP-1, and TRAP220 were translated in vitro with the TNT-coupled reticulocyte lysate system (Promega Corp.) from their mammalian expression plasmids described above. Then 8-μl volumes of the translated proteins were added to 96-well plates containing immobilized ERα, ERβ, or BSA and incubated at 4°C overnight. The wells were washed five times with PBST to remove unbound protein, and the bound protein was eluted by adding prewarmed (80°C) SDS-PAGE sample buffer and incubated at 80°C for 5 min. The supernatant was collected and boiled for 5 min before being separated on an SDS-polyacrylamide gel. The gel was dried, and the signals were detected by autoradiography.

RESULTS

Affinity selection of ligand-dependent ER binding peptides. The transcriptional activity of ER within target cells is influenced by its ability to interact with specific factors that decrease (corepressors) or increase (coactivators) its transcriptional activity (42). Over the past few years, the application of various molecular biology approaches has led to the discovery of co-activators that interact with the nuclear receptor HBD through a conserved LXXLL motif in a ligand-dependent manner. In this study, we used a combinatorial phage display approach to determine how flanking sequences influence the LXXLL motif-receptor interactions. The advantages of using this approach are twofold: a vast number of sequences can easily be assessed, and, more importantly, sequences obtained from this type of screening often reflect sequences that can be found in nature (35, 44). Specifically, a 19-mer phage "focused" library in which the LXXLL motif was flanked on each side by seven random amino acid residues was constructed. The resulting phage library was used to select for peptides that bound with high affinity to estradiol-activated ERα. Phage particles that bound specifically to ERα in a ligand-dependent manner were selected and amplified, and the amino acid sequences were deduced following DNA sequencing. Figure 1 shows representative peptide sequences derived from the isolated phage. Based on sequences flanking the core LXXLL motif, three different sequence clusters have emerged. Class I peptides contain a conserved serine at the −2 position and a positively charged residue (R) at the −1 position. Class II peptides have a proline occupying the −2 position and a hydrophobic leucine (L) residue directly preceding the LXXLL motif. Two of the three peptides in class II also contain a charged histidine (H) at the −3 position, and this histidine appears to have an influence on their binding characteristics (see Discussion). Class III peptides share a conserved serine (S) or threonine (T) at the −2 position followed by a hydrophobic leucine (L) or isoleucine (I) at the −1 position. In these initial characterizations, we used the intact bacteriophage to evaluate the ERα binding properties of these peptide sequences. To show that the peptide alone is both necessary and sufficient for ER binding, we subcloned representative members of each class of peptides as fusion proteins to bacterial alkaline phosphatase (50) and demonstrated that the purified recombinant peptide-enzyme fusions interacted specifically with ERα (data not shown).

We next developed a series of mammalian two-hybrid assays to confirm that the LXXLL-containing peptides identified could interact with ERα in the context of the intact cell. For this purpose, full-length ERα was expressed as a fusion protein with the VP16 acidic activation domain and the peptide sequences were produced as fusions with the yeast Gal4DBD. Interaction between ERα-VP16 and the LXXLL-Gal4DBD fusions was assessed by using the 5×Gal4Luc3 luciferase reporter gene, which contains five copies of the Gal4 responsive element upstream of a simple TATA box. Shown in Fig. 2 are comparisons of the abilities of different ligands to activate ERα transcription through a classical ER responsive element (Fig. 2A) and their ability to facilitate the interaction of the LXXLL peptide (class I-ER4) with ER (Fig. 2B). All steroidal and nonsteroidal ER agonists strongly activated transcription from the 3×-ERE-TATA-Luc reporter (Fig. 2A), while the SERMs 4-hydroxytamoxifen and GW7604 displayed minimal agonist activity within this promoter context (Fig. 2C). The pure antagonist ICI 182,780, as expected, functioned as an inverse agonist that suppressed the transcription below the basal, no-hormone treatment level (Fig. 2C). When analyzing the interaction between the LXXLL motif and ERα, we observed a low but significant basal level of interaction in the absence of any ligand treatment, indicating that some of the expressed ERα is already in an active conformation, allowing the LXXLL peptide to interact. At present, we do not know whether this basal activity is caused by residual estrogens present in the charcoal-stripped serum or is due to alternative pathways that activate

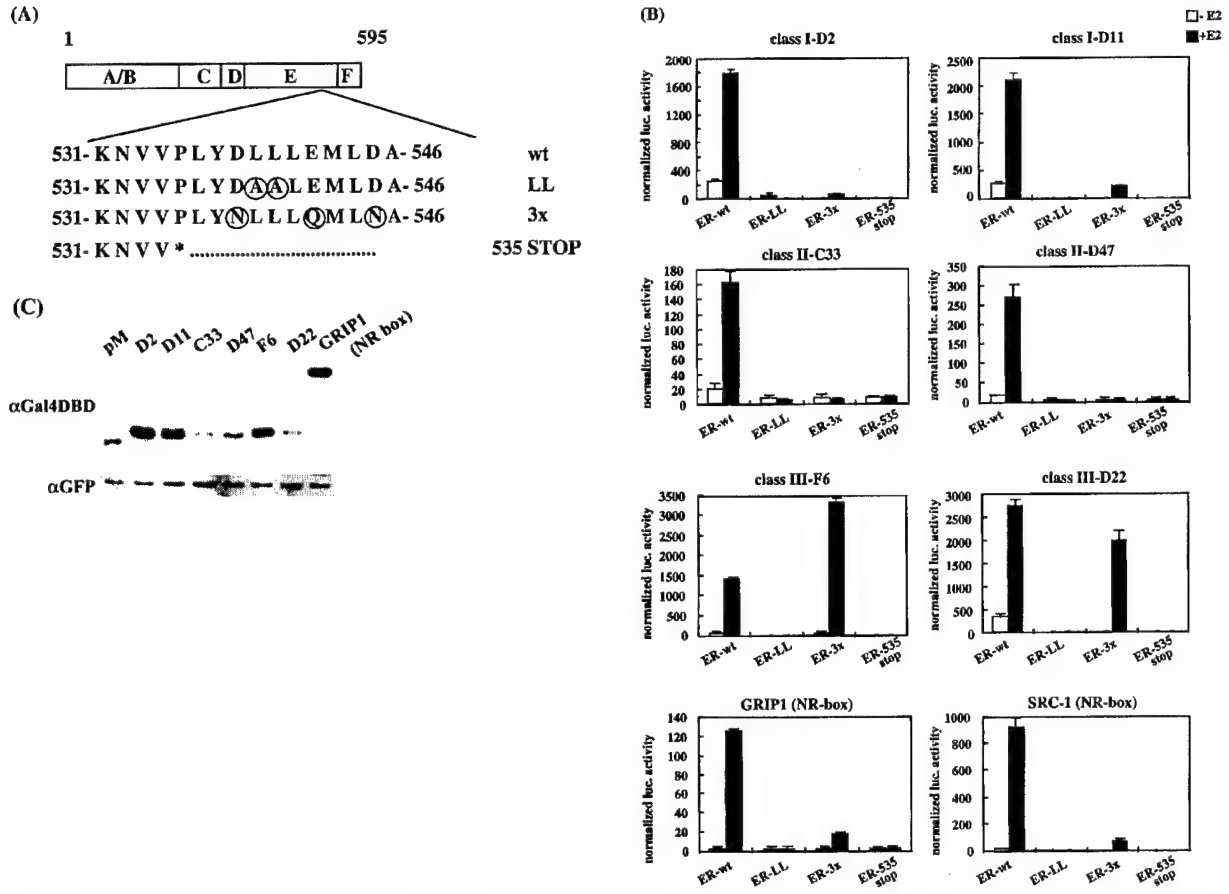
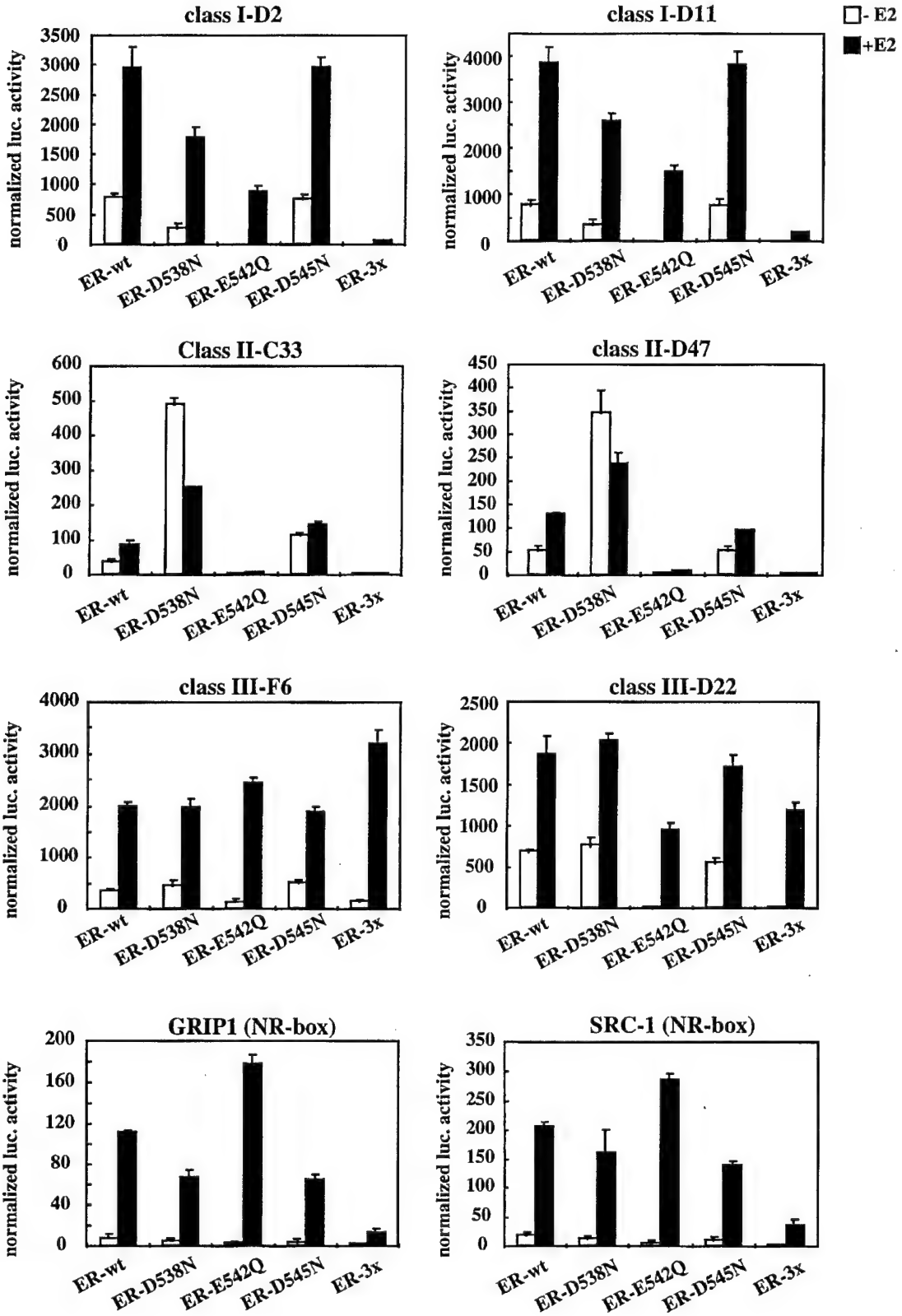


FIG. 3. Not all LXXLL peptide-ER interactions require a functional AF-2. The three groups of LXXLL-containing peptides interacted differentially with ER helix 12 mutants. (A) A schematic drawing of the wild-type (wt) ER is shown along with a region of the HBD corresponding to ER activation function 2 (AF-2). Residues that were mutated are indicated by circles. (B) Mammalian two-hybrid assays were used to test whether all the LXXLL motifs interacted with the same region of ER. Peptide sequences representing three LXXLL classes were expressed as fusion proteins to the Gal4DBD. Wild-type (wt) and mutant ERα were expressed as VP16 fusion proteins. The binding capacity of different peptides to wild-type and mutant ER was measured by using a 5×Gal4Luc3 reporter construct. GRIP-1 (NR-box) and SRC-1 (NR-box) constructs contain the center three copies of an LXXLL motif (amino acids 629 to 760 for GRIP-1 and 621 to 765 for SRC-1) fused to Gal4DBD. (C) Western analysis of the expression levels of selected Gal4DBD-peptide fusions. Nuclear extracts were prepared from transfected HeLa cells and analyzed using SDS-PAGE. The peptide-Gal4DBD fusion proteins were detected with a monoclonal antibody raised against Gal4DBD (αGal4DBD). The expression levels of the Gal4DBD fusions were normalized by assaying the levels of EGFP expressed from a cotransfected plasmid (pEGFP-C3). Specifically, the identical blot was reprobed with a polyclonal anti-GFP antibody (αGFP).

ER-mediated transcription. However, we observed that above the basal level, the interaction of the LXXLL peptide with ERα was entirely ER agonist dependent. The ability of both steroidal and nonsteroidal ER agonists to promote the ERα-LXXLL peptide interaction parallels the ability of these compounds to activate ERα-mediated transcription through a classical ER–ERE-mediated pathway. This indicates that all of these compounds are mechanistically similar, inducing similar conformational changes within ERα, and that within target cells these ligand-receptor complexes are likely to recruit the same coactivators. Interestingly, none of the ER antagonists or SERMs tested were able to facilitate ERα-LXXLL interactions. The pure antagonist ICI 182,780 totally abolished both basal peptide-ERα interactions and ERα-mediated transcription (Fig. 2C and D). In addition, although SERMs such as 4-hydroxytamoxifen and GW7604 can manifest partial agonist activity in certain cell types and promoter contexts (Fig. 2C and data not shown), in this experiment they actually drove the receptor into a conformation which prohibited LXXLL peptide-ERα interactions from occurring. As a result, the basal

FIG. 4. The interaction of ERα with each of the three classes of LXXLL peptides identified is affected differentially by helix 12 mutations. The contributions of each of the three charged residues (D538, E542, D545) within helix 12 to LXXLL motif-ERα interactions were evaluated. Specifically, we created single point mutations of each residue to their corresponding amides and evaluated the impact of these mutations on ERα-LXXLL peptide interactions in a mammalian two-hybrid assay. The mutants indicated were generated by site-directed mutagenesis within the wild-type (wt) VP16-ERα backbone. Selected peptide sequences representing each of the three LXXLL classes were expressed as Gal4DBD fusions. The binding capacity of the different peptides to wild-type and mutant ER was measured by using a 5×Gal4Luc3 reporter construct. GRIP-1 (NR-box) and SRC-1 (NR-box) constructs contain the center three copies of an LXXLL motif (amino acids 629 to 760 for GRIP-1 and 621 to 765 for SRC-1) fused to Gal4DBD.


class I-D2
class I-D11
- E2
+E2
Class II-C33
class II-D47
class III-F6
class III-D22
GRIP1 (NR-box)
SRC-1 (NR-box)
normalized luc. activity
ER-wt
ER-D538N
ER-E542Q
ER-D545N
ER-3x

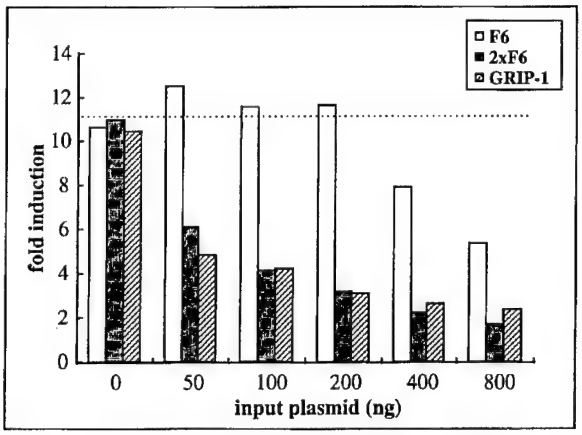


FIG. 5. LXXLL-containing peptides disrupt ERα transcriptional activity when overexpressed in target cells. HeLa cells were transfected with the ERα expression plasmid (pRST7ERα), 3×ERE-TATA-Luc reporter, along with increasing amounts of a construct expressing the peptide-Gal4DBD fusions as indicated. F6 contains a single copy of the F6 peptide, 2×F6 contains two copies of the F6 peptide with 50 amino acids separating the two LXXLL motifs, and GRIP-1 contains the center three NR boxes from the coactivator GRIP-1. All these peptides were expressed as fusion proteins to Gal4DBD. In addition, a pCMVβgal plasmid was cotransfected to normalize for transfection efficiency. After transfection, cells were induced with 10^{-7} M 17β-estradiol for 16 h before assaying. Fold induction represents the ratio of estradiol-induced activity versus no-hormone control for each transfection.

level of interaction between ERα and peptides containing the LXXLL motif was abolished in the presence of these compounds (Fig. 2D). The crystal structures of raloxifene-, tamoxifen-, and estradiol-activated ERα HBD have recently been solved and indicate that the coactivator binding groove within the receptor is occupied by a mispositioned helix 12 upon antagonist binding (4, 41). Helix 12 of the receptor thus prevents the coactivator LXXLL motif from interacting. Although some of our peptides seem to bind strongly to ERα in the presence of estradiol, none of them were able to interact with ERα in the presence of any of the SERMs tested, including 4-hydroxytamoxifen, nafoxidine, raloxifene, GW7604, and clomiphene (data not shown). Therefore, the partial agonist activity manifested by these compounds in some cells is likely to require cofactors distinct from those required by estradiol-activated ER (29). These data support the notion that the ability to facilitate the interaction of ER with LXXLL-containing coactivators is a fundamental step common to both ligand-dependent and basal transcriptional activity mediated by ERα. The observation that ER-peptide interactions do not occur in the presence of ER antagonists or mixed agonists may explain why compounds like tamoxifen and ICI 182,780 can inhibit both basal and ligand-dependent activation of ER. We also conducted the same analysis with other LXXLL-containing peptides and observed similar results (data not shown).

Not all LXXLL motifs are functionally equivalent. We next examined whether all of the LXXLL-containing peptides selected by using phage display were functionally equivalent. The previously defined ternary structures of the LXXLL motifs cocrystallized with either the ERα or PPARγ HBD indicated that these motifs bind to a hydrophobic groove created by helices 3, 4, 5, and require an intact helix 12 (26, 41). Therefore, the ability of the LXXLL motifs identified to interact with the coactivator binding groove was assessed by using a modified mammalian two-hybrid assay. Several ERα mutants with alterations in helix 12 as well as the wild-type ERα were produced as VP16 fusion proteins to test their ability to recruit LXXLL motifs (Fig. 3A). We found that all of the peptides

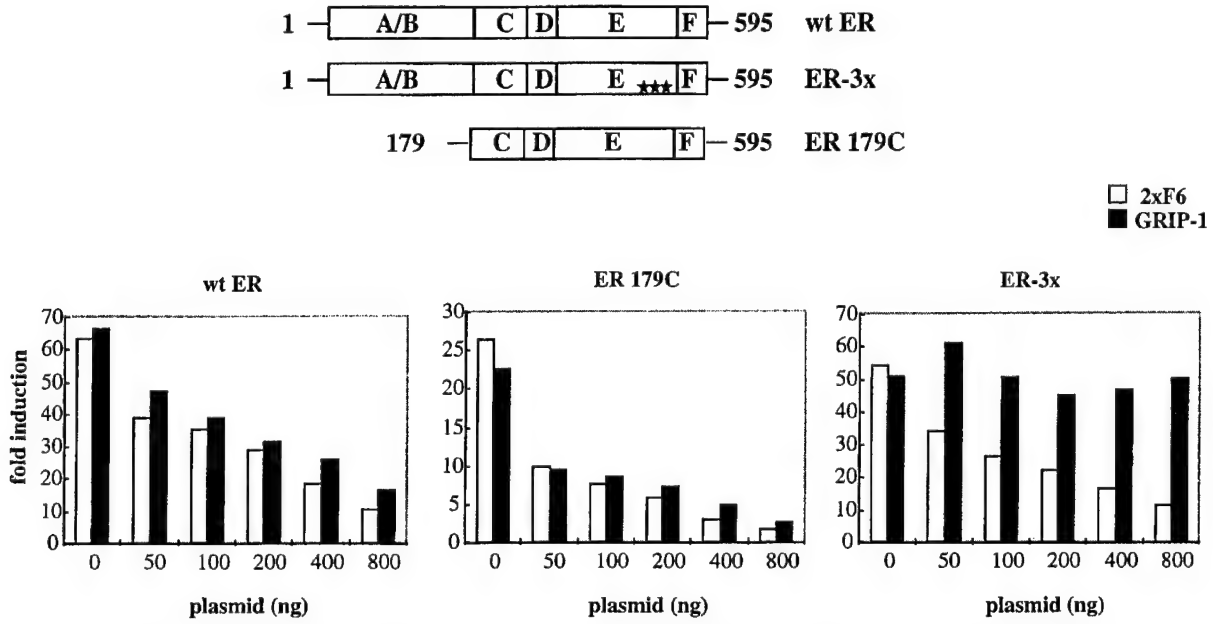


FIG. 6. The differential ability of LXXLL-containing peptides to disrupt ERα-mediated transactivation function reveals the presence of multiple ER-interacting coactivators. HepG2 cells were transfected with pRST7-ERα (wt), ERα179C, or ERα-3× mutant expression plasmids along with the 3×ERE-TATA-Luc reporter gene and increasing amounts of the Gal4DBD-peptide fusion constructs (as indicated). Fold induction represents the ratio of estradiol-induced (10^{-7} M) activity versus no-hormone control for each transfection.

tested interacted with wild-type ERα in a ligand-dependent fashion. As expected, the middle three copies of the LXXLL motif (NR box) found in the coactivators SRC-1 and GRIP-1 also interacted in a similar fashion (Fig. 3B, ER-wt). Western analysis showed that different classes of peptide-Gal4DBD fusion proteins have different expression levels in the cells; therefore, the data presented in this assay can be used to compare only their binding patterns, not their relative binding affinities (Fig. 3C). For instance, the class II peptides interacted with ERα with relatively higher affinity than did the class I and III peptides in the in vitro binding assays (data not shown). The expression levels of these peptides, however, are much lower than those of the other classes of peptides, which may explain the observed lower readout in the mammalian two-hybrid assays. Regardless, the mammalian two-hybrid assay remains a useful tool to characterize the in vivo interactions between ERα and the peptides.

Truncation of ER helix 12 (ER535 stop) does not affect ligand binding or dimerization; however, the ability of the receptor to interact with any LXXLL peptides was totally abolished. This was consistent with the observation that helix 12 is required to form the coactivator binding groove, and, more importantly, it implied that all the affinity-selected LXXLL-containing peptides bind to the same coactivator binding groove. Furthermore, mutation of a pair of the hydrophobic residues in helix 12 (L539L540→A539A540) significantly decreased the ERα transcriptional activity and also abolished the interaction of ERα with all of the LXXLL peptides tested (ER-LL in Fig. 3B).

Previously, we and others have demonstrated that alteration of the three charged residues in ERα helix 12 (D538E542D545→N538Q542N545; ER-3×) abolishes ERα transcriptional activity in most cell types (10, 28, 47) and prevents the interaction of GRIP-1- and SRC-1-type coactivators with ERα. Predictably, in our experiments, the interaction of the ERα-3× receptor mutant with the GRIP-1 and SRC-1 NR boxes was significantly lower than that of the wild-type receptor (Fig. 3B, ER-3×). The ability of class I and II peptides to interact with ERα was also prevented by these specific ERα helix 12 mutations, indicating that they may bind to ERα in a manner which is similar to that of the GRIP-1 and SRC-1 LXXLL motifs. In contrast, the interactions between class III peptides and ERα was not affected by these mutations. Importantly, the ERα-3× mutant is fully functional in certain cell types, which is interesting in light of the observed weak interaction of this receptor with coactivators like SRC-1 and GRIP-1. Our observations suggest, however, that the activity exhibited by this mutant receptor might be the result of its interaction with cofactors containing class III type LXXLL motifs. Regardless, however, it appears that the LXXLL motif is not merely a receptor-cofactor docking sequence but also contains information that governs the specificity of these interactions.

To further characterize the interactions between ERα and these three classes of peptides, we made individual mutations within the ER-3× to evaluate the relative contributions of each of the three charged residues (D538, E542, and D545) in ER-LXXLL motif interactions. This analysis revealed that the diminished interaction of class I peptides with ER-3× seems to be the sum of changing Asp-538 and Glu-542 to their corresponding amides; however, the change of Glu-542 to Gln-542 had the greatest impact on this interaction (Fig. 4). Glu-542 also appears to be the most important residue in determining the interaction between ERα and class II peptides, since mutation of this residue led to a total loss of interaction. Interestingly, changing Asp-538 to Asn-538 increased the binding of ERα with the class II peptides; however, this was observed to occur in a ligand-independent manner. Predictably, none of the mutations appear to have affected the ability of ERα to recruit class III peptides, consistent with the notion that ERα might interact with this class of peptides in a specific manner. The interaction patterns of ERα with GRIP-1 and SRC-1 NR boxes are similar to each other, in that none of the individual residue changes had a significant impact on the strength of the interaction. Replacing all three residues, however, greatly reduced the ability of ERα to bind to these NR boxes. The precise mechanism of interaction of ERα with these peptides can be resolved only by studying the cocrystal structure of these complexes. The results of these assays, nevertheless, once again highlight the fact that not all LXXLL motifs interact with ERα in the same manner.

LXXLL-containing peptides can disrupt ERα transcriptional activity in the target cells. If peptides obtained from phage display are in fact mimicking the interactions between ERα and endogenous cofactors, they should function in a dominant negative manner when coexpressed in cells, disrupting these interactions and blocking the ER transcriptional activity. Coexpression in HeLa cells of the peptide F6-Gal4DBD fusion did indeed decrease the estradiol-induced ER-dependent reporter gene expression to approximately 50% of that without the peptide (Fig. 5, F6). We have also tested other peptides from all three classes and found that all the LXXLL peptides we obtained were able to disrupt ER transcriptional activity in a similar manner (data not shown). It was suggested previously (26) that multiple copies of the NR boxes in GRIP-1 and SRC-1 can bind to ERα in a synergistic manner. Thus, as expected, expression of the center three copies of the NR boxes from GRIP-1 permitted a more effective inhibition of ER-mediated transcription than did expression of a single-copy peptide (Fig. 5, compare F6 and GRIP-1). Based on this result, we examined the inhibitory activity of a construct expressing two copies of the LXXLL motif on ERα transcriptional activity. The linker between the two copies was adapted from sequences found between the GRIP-1 NR box 2 and NR box 3 (see Materials and Methods). When analyzed in target cells, the fusion proteins containing two copies of the F6 peptide were more effective inhibitors of ERα transcriptional activity than were those expressing a single copy. 2×F6 was functionally comparable to the construct expressing the GRIP-1 NR boxes, which contains three copies of the LXXLL motif (Fig. 5, 2×F6). The increased efficacy of 2×F6 as an inhibitor of ER function required each of the two LXXLL motifs, since addition of the GRIP-1 linker sequence to a single copy of F6 did not increase its antagonist efficacy (data not shown).

It has been demonstrated by us and others that ER contains two distinct activation function domains, AF-1 and AF-2, whose activities are manifested in a cell-selective manner (3, 34, 45, 47). Both AF-1 and AF-2 functions are required for maximal ER transcriptional activity in HeLa cells, while AF-1 is the dominant activation function in HepG2 cells. Our peptide disruption results closely correlated with these observations. In HeLa cells, overexpression of LXXLL-containing peptides abolished almost 100% of the ER transcriptional activity (Fig. 5), highlighting the obligate role of AF-2 in ER-mediated function and showing that AF-1 is not able to function independently of AF-2 in this background. However, we have observed that the roles of AF-1 and AF-2 in HepG2 cells are different. It was demonstrated in a previous study that mutations in ER-AF2 that block the binding of the coactivators SRC-1 and GRIP-1 with ER have no effect on ER transcriptional activity in HepG2 cells (19, 28, 47). We interpreted these

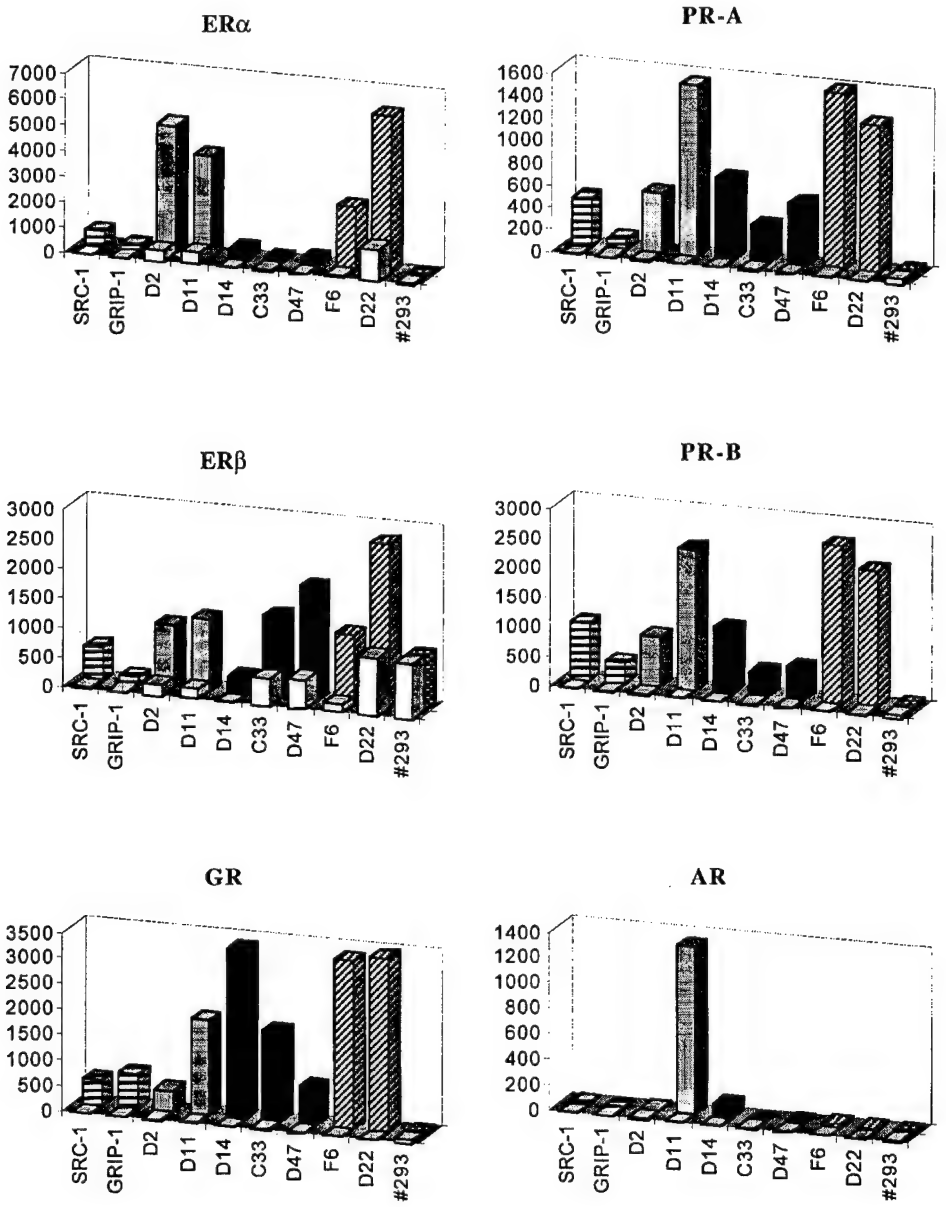


FIG. 7. Nuclear receptors have distinct preferences for different LXXLL motifs. The interactions between different LXXLL motifs and nuclear receptors were assayed by using a mammalian two-hybrid system. Full-length receptors and selected peptides were expressed as VP16 and Gal4DBD fusion proteins, respectively. The magnitude of these interactions was measured by using a 5×Gal4Luc3 reporter gene. Open bars, no hormone; hatched or filled bars, hormone treatments. The following hormones were used in this experiment: 10^{-7} M 17β-estradiol for ERα and ERβ, 10^{-7} M progesterone for PR-A and PR-B, 10^{-7} M dexamethasone for GR, 10^{-7} M 9-*cis*-retinoic acid for RARα and RXRα, 10^{-7} M T3 for TRβ, 10^{-7} M 1,25-dihydroxyvitamin D_3 for VDR, and 10^{-6} M 5α-dihydrotestosterone for AR. The luciferase activity was normalized to the activity of the cotransfected pCMVβgal.

data to mean that either (i) in this context AF-1 is dominant and AF-2 is not required or (ii) in this cell line a cofactor exists whose interaction with ER does not require an intact AF-2. To discriminate between these possibilities, we used the LXXLL-containing peptides to study the role of AF-1 and AF-2 in ER signaling in this background. The results of this analysis are shown in Fig. 6. When either the 2×F6 or GRIP-1 peptides were overexpressed in HepG2 cells, they inhibited wild-type ER transcriptional activity; however, it was not inhibited down to the basal levels (Fig. 6, wt ER). The transcriptional activity was still about 10-fold over the basal levels at the highest dose of input peptide fusion plasmid, indicating that some independent AF-1 activity is possible in this cell context. This hypothesis is supported by the observation that the activity of an ER-mutant lacking AF-1 was inhibited more readily (twofold over the basal level at the highest input plasmid dose) by overexpression of either of the peptide fusions (Fig. 6, ER 179C). The most interesting result, however, was that the class

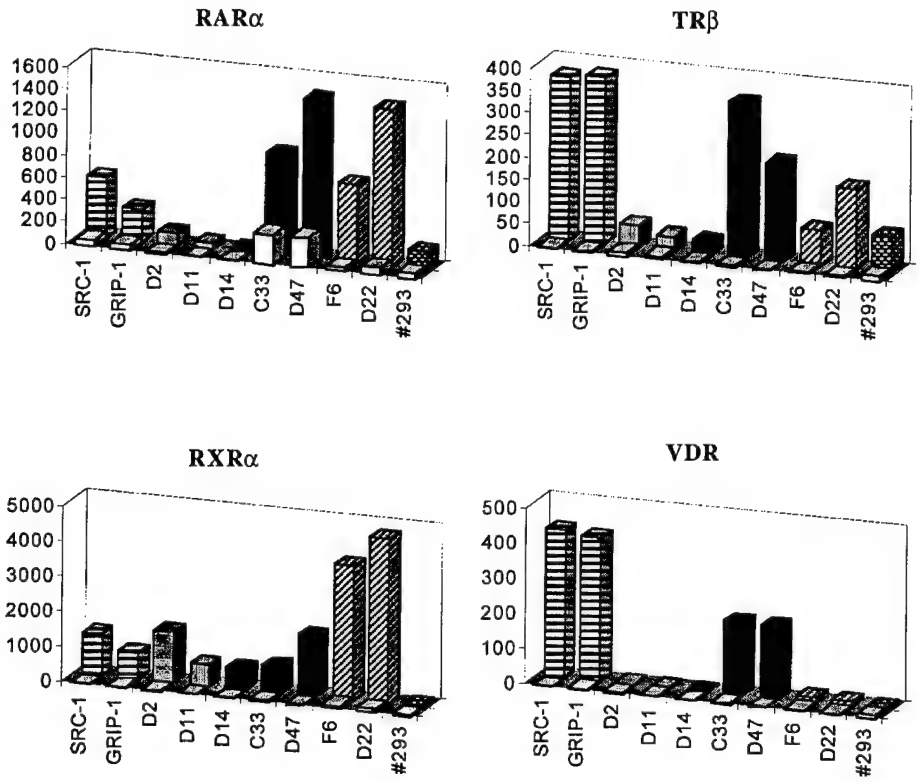


FIG. 7—*Continued.*

III peptide (2×F6) was an efficient inhibitor of ER-3× transcriptional activity whereas the GRIP-1 NR-box peptide was inefficient (Fig. 6, ER-3×). Taking these results together, we observed that the class III peptide F6 interacted with ER-3× (Fig. 3B) and that overexpression of this peptide inhibited the transcriptional activity of this mutant receptor, suggesting that a cofactor which contains an F6-like LXXLL motif may exist in HepG2 cells and may be important for ER function.

Sequences flanking the LXXLL core motif influence receptor selectivity. The GRIP-1 and SRC-1 coactivators containing multiple LXXLL motifs interacted with most nuclear receptors. Alterations of residues surrounding these motifs have been shown to affect receptor selectivity; therefore, we next wished to define the sequences within the NR box which enable it to discriminate between receptors by using the LXXLL-containing peptides identified. For this study, we used representative members of each class of LXXLL identified from our focused library along with an LXXLL motif, #293, which was identified previously in screens of random peptide libraries for peptides which interacted with estradiol-activated ERβ (reference 32 and data not shown). This specificity analysis was accomplished by performing mammalian two-hybrid assays, in which the LXXLL-containing peptides were fused to Gal4DBD and the full-length receptors were expressed as VP16 fusion proteins. As shown in Fig. 7, most steroid receptors interacted with all three classes of peptides efficiently. The lower luciferase activity observed with class II peptides is probably related to the lower (~10-fold) expression level of this class of peptides (Fig. 3C). Regardless, the RXR heterodimerization partners, such as RARα, TRβ, and VDR, demonstrated a strong preference for class II over the other classes of peptides. Interestingly, ERβ also showed the same tendency, preferring to interact with class II motifs, suggesting that the coactivator binding groove in ERα and ERβ may be functionally different. Interestingly, with the exception of D11, the AR interacted weakly with all the LXXLL peptides tested, sup-

TABLE 1. Each of the three classes of ER-interacting LXXLL motifs is found within known coactivators

Class	Coactivator	Sequence[a]
Class I		SRLXXLL
	GRIP1	TKLLQLL
	SRC-1	HKLVKLL
	AIB-1	KKLLQLL
Class II		PΦLXXLL
	TRAP220	PILTSLL
		PMLMNLL
	RIP140	PILYYML
Class III		(S/T)ΦLXXLL
	RIP140	TYLEGLL
		TLLASLL
		SLLLHLL
		TLLQLLL
		TVLQLLL
	PGC-1	SLLKKLL
	DAX-1	SILYNLL
		SILYSML
		SILYSLL
	SHP	TILYALL
		SILKKIL

[a] X, any amino acid; Φ, hydrophobic amino acid. Conserved amino acids in each class are in boldface type.

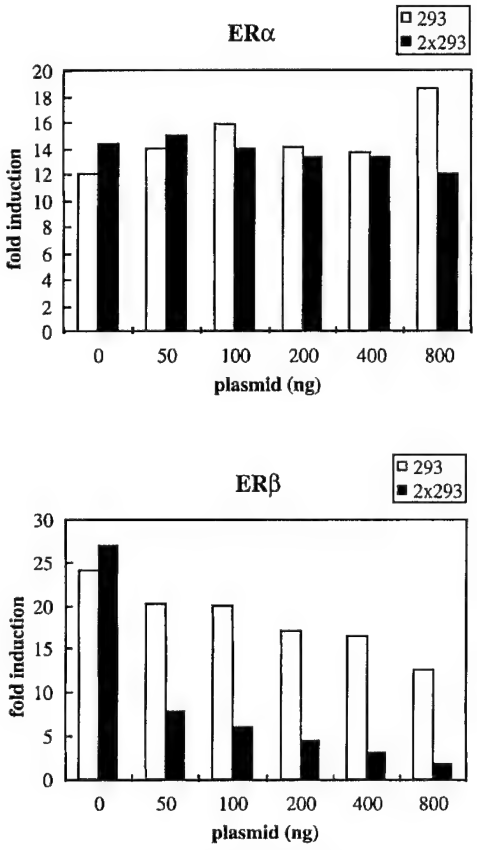


FIG. 9. Peptide #293 selectively disrupts ERβ-dependent reporter gene expression without affecting ERα-mediated transcription when expressed in target cells. Peptide #293 containing an LXXLL motif was affinity selected by phage display with estradiol-activated ERβ as a target. Expression of either one copy or two copies of this peptide did not interfere with the transcriptional activity of ERα but disrupted ERβ-mediated transcriptional activity. HeLa cells were transfected with either ERα or ERβ expression plasmids, along with 3×ERE-TATA-Luc reporter, pCMVβgal, and increasing amounts of Gal4DBD-peptide fusion constructs as indicated. Fold induction represents the ratio of activity estradiol-induced activity versus no-hormone control for each transfection.

porting the hypothesis that alternative coactivator recruitment methods are used by AR and that the N terminus is more important than AF-2 in recruiting coactivators to the receptor (2, 33, 51).

We next compared the sequences of these three classes of LXXLL-motifs with sequences of NR boxes in known coactivators and found that the class I peptides share similar features with two of the LXXLL motifs found in GRIP-1- and SRC-1-type (p160s) cofactors, in which a positively charged residue precedes the LXXLL motif (Table 1). The class II peptides were represented by the two LXXLL motifs found in TRAP220 (52), in which a proline occupies the −2 position. The class III peptides are most abundant in cofactor RIP140 (6), but similar motifs can also be found in PGC-1 (36), and the orphan receptors SHP and DAX-1 (39, 53). Based on our findings, we predicted that each of these cofactors should interact with both isoforms of ER. These factors have already

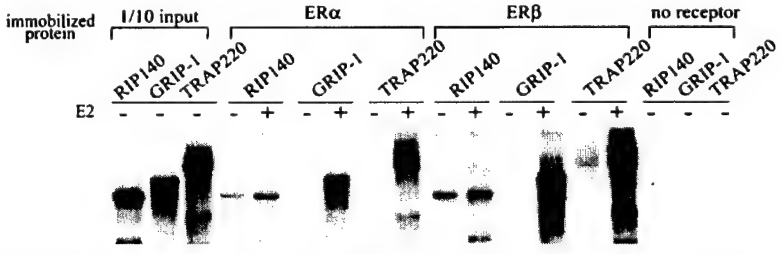


FIG. 8. LXXLL motif-containing cofactors interact with both ERα and ERβ in vitro in a ligand-dependent manner. Equal amounts of full-length ERα, ERβ, or control BSA were immobilized on 96-well plates in the presence or absence of 1 μM estradiol. Full-length RIP140, GRIP-1, and TRAP220 were translated in vitro and labeled with [^{35}S]methionine. Labeled cofactors were added to the wells containing immobilized protein and incubated at 4°C overnight. Unbound protein was removed by washing, and the bound protein was eluted, separated by SDS-PAGE, and visualized by autoradiography.

been shown to interact with ERα, whereas minimal information on their ERβ binding properties has yet to be reported. In a pulldown assay with purified full-length ERα and ERβ immobilized on 96-well plates, we were able to confirm that each of these proteins, representing all three LXXLL classes, was able to interact with both ER isoforms in a ligand-dependent manner (Fig. 8).

Peptide #293 is an ERβ-selective antagonist. When peptide #293 was screened against a panel of nuclear receptors, it showed a strong preference for ERβ and interacted weakly with TRβ and RARα but did not interact significantly with the other receptors tested (Fig. 7). Thus, receptor specificity can be achieved by altering sequences flanking the core LXXLL motif, and it is possible that ERβ-specific coactivators will be found to contain this or a structurally similar motif. To test whether peptide #293 could specifically target ERβ transcriptional activity, we overexpressed it as a Gal4DBD fusion protein and assayed its ability to disrupt ERβ-dependent reporter gene expression. As shown in Fig. 9, expression of #293 had no effect on ERα-mediated gene expression but the ERβ transcriptional activity was significantly reduced. Similar to the results with ERα, two copies of the #293 motif (2×293) disrupted ERβ function more efficiently than did a single-copy peptide. Nevertheless, ERα transcriptional activity remained unaffected by the expression of 2×293. Clearly, not all LXXLL motifs have the same receptor binding selectivity. Thus, we believe that receptor-specific LXXLL motifs can be found and used to target specific cofactor-receptor interactions.

DISCUSSION

The identification of ER-associated coactivators and corepressors has helped us understand how different ligands acting through the same receptor can manifest different biological activities. The importance of these proteins in mediating ER pharmacology was highlighted by our previous studies, which described the identification of different classes of peptides whose ability to interact with ER is influenced by the nature of the bound ligand (29, 32). All of these interactions represent potential ER-cofactor interactions and suggest that ER pharmacology is more complex than was originally believed. In this study, we have focused on one receptor binding motif, LXXLL, and have demonstrated that even within this specific core there are multiple classes of functionally different LXXLL motifs. Using estradiol-activated ERα, we screened 10^8 variations of the LXXLL motif and identified three classes of peptides that interact with the coactivator binding pocket within the ERα HBD. The classifications were further substantiated by studies which revealed that each class of peptide displayed specific receptor preferences and that their binding to ERα was differentially affected by ER helix 12 mutations. In spite of their differences, the LXXLL-containing peptides all appear to bind in an agonist-dependent manner to the same coactivator binding groove within ERα HBD. None of the peptides identified interact with ER-535stop (helix 12 deletion) or the LL mutant (L539L540→A539A540). This is not surprising, since the cocrystal structure of ER with NR box 2 of GRIP-1 shows that several residues in helix 12, including L-539, are required to make van der Waals contacts between the coactivator groove and the LXXLL peptide. It is likely that truncation of helix 12 or mutations of the paired hydrophobic residues destabilize such interactions. Furthermore, replacing the three charged residues in helix 12 with their corresponding amides (ER-3×) disrupts the ability of class I and class II peptides to interact with ER. The ternary structure predicted from the cocrystal structure suggests that the conserved glutamic acid (E542) in ER helix 12 plus the lysine residue (K362) in helix 3 cap the LXXLL peptide in the coactivator binding groove through hydrogen bonding to the backbone amides or carbonyls of the residues on the N- or C-terminal turns of the peptide helix. Although the charged side chain is not directly involved in the hydrogen bonding, the positively charged residue preceding the LXXLL motifs is thought to be important for orienting and positioning these motifs within the coactivator binding groove, which is capped on one end by the negatively charged E-542 (26, 41). Consistent with this idea, our results showed that changing the Glu-542 into Gln-542, which neutralizes the charge but still preserves the hydrogen bonding, greatly reduced the ability of this mutant receptor to interact with class I and class II peptides. One of the most surprising findings of our study, however, is that the class III peptides, which do not contain any positively charged residues immediately preceding the LXXLL motif, interact strongly with both wild-type ER and the ER-3× mutant, supporting the hypothesis that this class of peptides binds in a unique manner to the ER AF-2 and that the "charged-clamp" model may not hold for all LXXLL interactions.

Because of the unique properties of the class III LXXLL, we searched the sequences of known nuclear receptor-interacting motifs for analogous sequences. Interestingly, class III-like LXXLL motifs were found to be present in multiple copies in RIP140, where the LXXLL motifs are preceded by a serine or threonine and an isoleucine or leucine. Importantly, RIP140 was shown to interact with ER-3× (6), whereas GRIP-1 and SRC-1 did not, suggesting that the class III peptides represent a biologically relevant LXXLL motif. Similar types of motifs were also found in the orphan receptors DAX-1 and SHP (39, 53), two receptors that are able to interact with estradiol-activated ER and disrupt its ability to activate transcription. Although the domains within DAX-1/SHP responsible for these interactions have not been precisely determined, based on their interaction patterns (induced by estradiol, inhibited by tamoxifen, and insensitive to ER-3× mutations), we anticipate that these interactions are mediated, at least in part, through LXXLL-like motifs. Since both RIP140 and SHP can disrupt wild-type- as well as ER-3× mutant-mediated transactivation (references 18 and 40 and data not shown), it is tantalizing to speculate that class III type motifs might be used by ER inhibitors instead of ER coactivators. We were able to show, however, that the F6 peptide (class III) can compete with endogenous cofactors and suppress estradiol-induced ER activation in target cells. This leaves open the possibility that another class of receptor coactivators that use the class III-like LXXLL motif remains to be found. Clearly, not all LXXLL motifs are the same. However, until each of these motifs is found within a bona fide ER regulator, the functional significance of these different peptides cannot be determined. Regardless, our study highlights a heretofore unanticipated complexity in ER action.

All of the AF-2-interacting coactivators that have been found contain an LXXLL motif. Thus, given the homology in the AF-2 domain among receptors and the simplicity of the LXXLL motif, it was difficult to understand how receptor specificity could occur. Interestingly, with the collection of peptides we obtained, we were able to demonstrate that ERα and ERβ, two highly homologous receptors with similar ligand binding characteristics, showed distinct preferences for different classes of peptides. Previously, we found that the ERβ homodimer is a weaker transcriptional activator than the ERα homodimer and the ERαβ heterodimer (14). It would be interesting to see if the differences in their transcriptional activity are due to their differential association with different cofactors.

Although ERα and ERβ have overlapping affinities for their ligands and DNA responsive elements, they are not functionally redundant (22, 31). Their ability to interact differentially with different LXXLL motifs within coactivators might explain how ERα and ERβ manifest different transcriptional activities in target cells.

The PPARγ-binding protein (54) and its human homolog TRAP220 (also called DRIP205) (37, 52) contain LXXLL motifs that have a proline at the −2 position, similar to the class II peptides. These cofactors were identified originally by their ability to interact with PPARγ, TR, and VDR in vivo and were shown to interact with RAR and RXR at high affinity in vitro. A remarkably similar pattern was observed in our study when we demonstrated by mammalian two-hybrid analysis that TR, VDR, RAR, and ERβ appeared to have a stronger preference for the class II peptides, suggesting that the occurrence of a proline at the −2 position might favor these interactions. Based upon alanine scanning studies, McInerney et al. suggested that receptor recognition is most probably contributed by residues C-terminal to the LXXLL motifs (25). In our study, however, we did not find a good consensus in the C terminus in over 50 peptides selected from both random and focused library screening, using either ERα or ERβ as the target (Fig. 1) (reference 32 and data not shown). In contrast, residues at the −2 and −1 positions are dominated by either S(R or K) or S(I or L), which suggests that residues in these positions are important for cofactor-ER interaction through the LXXLL motif and that these sequences are generally accepted by steroid hormone receptors. Moreover, certain receptors such as TR, VDR, RXR, and ERβ appear to favor motifs with a proline at the −2 position, again highlighting the importance of this residue for receptor-cofactor recognition. However, we cannot rule out the possibility that the differences observed reflect a selection bias, since we have used only ER as a target for affinity selection. We would also like to emphasize that although residues occupying the −1 and −2 positions seem to be a critical determinant of LXXLL specificity, sequences outside these regions are also important, since a different receptor binding specificity has also been observed within the same class of peptides. For example, the ERβ-specific #293 peptide may be considered a class II member, because it also contains a proline at the −2 position. Clearly, however, sequences in addition to the proline at −2 are important, since #293 has a unique receptor selectivity.

The identification of novel classes of LXXLL motifs and the finding that they interact with ER in different ways have highlighted the complexity of ER action. As yet, given the limited number of coactivators and corepressors available for analysis, it is not possible to evaluate the full significance of our findings. However, we believe that these studies provide a glimpse of what is to come. In addition to the mechanistic insight offered by these studies, they have provided some novel technology which may be used in drug discovery. Some investigators have used the coactivator receptor ligand assay (CARLA) as a way of screening for compounds which function as receptor agonists and allow the formation of an AF-2/coactivator groove (21). For known receptors, where the cofactor interactions have been well established, this is likely to be useful. However, when studying an orphan receptor for which no ligand has been identified, its success relies on whether the receptor can interact with the coactivator chosen. For this purpose, a "universal" coactivator is desirable. Our studies have illustrated that several different LXXLL motifs interact differentially with different receptors. Therefore, the use of a single peptide in a screening paradigm can be risky, but the chance of success will be increased by incorporating several different classes of peptides in the screen.

Another application of these peptides, validated in our study, is their use as peptide antagonists of receptor function. For instance, peptide #293, when introduced into cells, specifically inhibits ERβ-mediated responses to estrogen. Since a specific small-molecule inhibitor of ERβ has not been identified, we believe that the #293 peptide may allow us to unravel some of the biology of this receptor. We believe that the technology used in our studies will also be useful for the study of orphan receptors. Specifically, we suggest that the identification of peptides which bind specifically to an orphan receptor and which inhibit its transcriptional activity can be used as "peptide antagonists" to study the biology of the receptor when its ligands are not known.

The results presented in this study confirm that the coactivator LXXLL motif is necessary and sufficient for receptor interaction. In addition, they revealed the importance of sequences surrounding the LXXLL core in determining receptor selectivity and in defining the manner in which coactivators interact with the nuclear receptors. The complexity highlighted by these studies suggests that the currently available coactivators and corepressors represent only a fraction of those which will ultimately be found and shown to interact with the nuclear receptors.

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Peptide Antagonists of the Human Estrogen Receptor

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Estrogen receptor α transcriptional activity is regulated by distinct conformational states that are the result of ligand binding. Phage display was used to identify peptides that interact specifically with either estradiol- or tamoxifen-activated estrogen receptor α. When these peptides were coexpressed with estrogen receptor α in cells, they functioned as ligand-specific antagonists, indicating that estradiol-agonist and tamoxifen–partial agonist activities do not occur by the same mechanism. The ability to regulate estrogen receptor α transcriptional activity by targeting sites outside of the ligand-binding pocket has implications for the development of estrogen receptor α antagonists for the treatment of tamoxifen-refractory breast cancers.

About 50% of all breast cancers express the estrogen receptor α (ERα) protein and recognize estrogen as a mitogen (*1*). In a subpopulation of these tumors, antiestrogens, compounds that bind ER and block estrogen action, effectively inhibit cell growth. In this regard, the antiestrogen tamoxifen has been widely used to treat ER-positive breast cancers (*2*). Although antiestrogen therapy is initially successful, most tumors become refractory to the antiproliferative effects of tamoxifen within 2 to 5 years. The mechanism by which resistance occurs is controversial; however, it does not appear to result as a consequence of ER mutations or altered drug metabolism (*3*). It may relate instead to the observation that tamoxifen is a selective estrogen receptor modulator (SERM), functioning as an ER agonist in some cells and as an antagonist in others (*4*). Consequently, the ability of tumors to switch from recognizing tamoxifen as an antagonist to recognizing it as an agonist has emerged as the most likely cause of resistance. Upon binding ER, both estradiol and tamoxifen induce distinct conformational changes within the ligand-binding domain (*5*). The tamoxifen-induced conformational change may expose surfaces on the receptor that allow it to engage the general transcription machinery. We used phage display to identify specific peptides that interacted with the estradiol- and tamoxifen-ER complexes and used these peptides to show that estradiol and tamoxifen manifest agonist activity by different mechanisms.

Affinity selection of phage-displayed peptide libraries was performed to identify peptides that could interact specifically with the agonist [17β-estradiol (estradiol) or 4-OH tamoxifen (tamoxifen)], activated ERα, or ERβ (*6*). Representative peptides from each of four classes presented in this study are shown in Fig. 1A. Several peptides that were isolated with estradiol-activated ERα (represented by α/β I) contained the Leu-X-X-Leu-Leu motif found in nuclear receptor coactivators (*7*). α II was isolated with either estradiol- or tamoxifen-activated ERα. Two classes of peptides, α/β III and α/β V, that interact specifically with tamoxifen-activated ERα and ERβ, respectively, were identified. The α/β V peptide was subsequently shown to interact with tamoxifen-activated ERα (*6*). Several additional peptides homologous to α/β V were identified. A BLAST search of the National Center for Biotechnology Information database with the derived consensus of the α/β V peptide class revealed that the yeast protein RSP5 and its human homolog, receptor potentiating factor (RPF1), both contain sequences homologous to α/β V. These proteins were previously shown to be coactivators of progesterone receptor B (PRB) transcriptional activity (*8*).

Peptide-peptide competition studies were performed with time-resolved fluorescence (TRF) to determine if the α II, α/β III, and α/β V peptides were binding the same or distinct "pockets" on the tamoxifen-ERα complex (*9*). The α/β III and α/β V peptides cross compete, and at equimolar peptide concentrations, 50% inhibition is observed (Fig. 1B). This result indicates that these two peptides bind to the same or overlapping sites on ERα. We believe that the α II peptide binds to a unique site as its binding was not competed by α/β V and only 50% inhibited by a 10-fold excess of the α/β III peptide.

We next assessed whether the peptides interacted with ERα in vivo using the mammalian two-hybrid system (*10*). The α/β I peptide interacted with ERα in the presence of the agonist estradiol but not the SERMs tamoxifen, raloxifene, GW7604, idoxifene, and nafoxidine or the pure antagonist ICI 182,780 (Fig. 2). The failure of antiestrogen-activated ERα to interact with the α/β I peptide is consistent with previ-

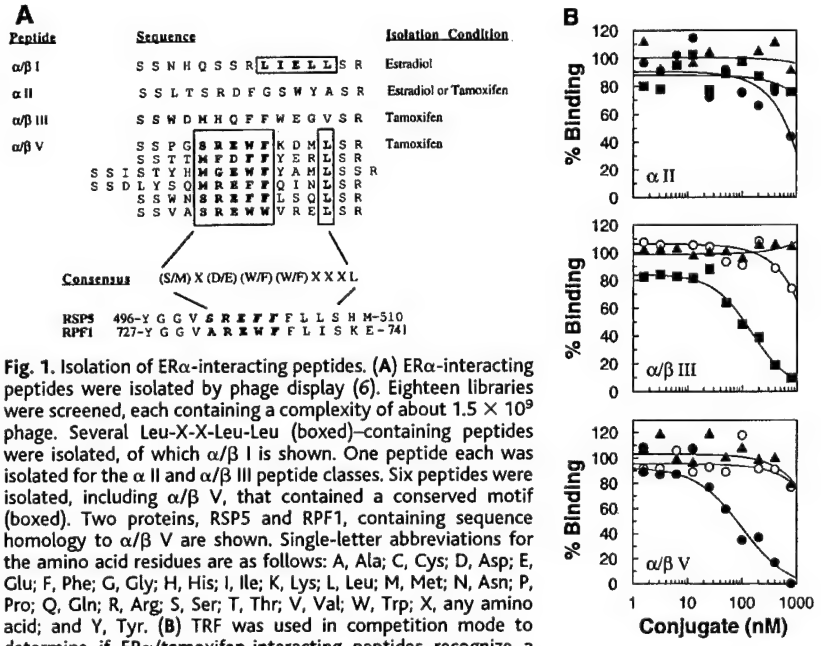

Fig. 1. Isolation of ERα-interacting peptides. (**A**) ERα-interacting peptides were isolated by phage display (*6*). Eighteen libraries were screened, each containing a complexity of about 1.5×10^9 phage. Several Leu-X-X-Leu-Leu (boxed)–containing peptides were isolated, of which α/β I is shown. One peptide each was isolated for the α II and α/β III peptide classes. Six peptides were isolated, including α/β V, that contained a conserved motif (boxed). Two proteins, RSP5 and RPF1, containing sequence homology to α/β V are shown. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; and Y, Tyr. (**B**) TRF was used in competition mode to determine if ERα/tamoxifen-interacting peptides recognize a common site on ERα (*9*). The peptide conjugate used for detection is indicated in each graph with the competing peptides as follows: ▲, no competitor; ○, α II; ●, α/β III; and ■, α/β V.

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ous studies that predict that the molecular mechanism of antagonism results from a structural change in the receptor ligand-binding domain that prevents coactivators from binding (*5*). α II interacted with the receptor in the presence of all modulators tested, with the unliganded (vehicle) and ICI 182,780–bound receptors showing the least binding activity. α/β III and α/β V interacted almost exclusively with the tamoxifen-bound ERα. ERα did not interact with the Gal4 DNA-binding domain (DBD) (control) alone in the presence of any modulators tested. Further studies indicated that binding of α II, α/β III, and α/β V occurs within the hormone-binding domain between amino acids 282 and 535 (*11*) and, unlike binding of α/β I, does not require a functional activation function 2 (AF-2) (www.sciencemag.org/feature/data/1039590.shl). These data indicate that SERMs induce different conformational changes in ERα within the cell and firmly establish a relation between the structure of an ERα-ligand complex and function.

When we examined the specificity of interaction between the peptides and heterologous nuclear receptors, we found, as expected, that the α/β I peptide interacted with ERβ, PRB, and the glucocorticoid receptor (GR) when bound by the agonists estradiol, progesterone, and dexamethasone, respectively (Fig. 3, A, B, and C). The α/β V peptide interacted with tamoxifen-bound ERβ and unexpectedly with PRB in the presence of the antagonists RU 486 or ZK 98299 (Fig. 3, A and B). The α/β V peptide, however, did not interact with the GR when bound by RU 486 or ZK 98299. α II and α/β III peptides failed to interact with ERβ, PRB, or GR.

We next tested the ability of the peptide-Gal4 fusion proteins to inhibit ERα transcriptional activity. Tamoxifen displayed partial agonist activity when analyzed with the ER-responsive complement 3 (C3) promoter in HepG2 cells (Fig. 4A). This activity can reach 35% of that exhibited by estrogen and is mediated by three nonconsensus estrogen response elements (EREs) located in the C3 promoter (*12*). When expressed in this system, the α/β I and α II peptides inhibited the ability of estradiol to activate transcription up to 50% and 30%, respectively (Fig. 4B). Two copies of the Leu-X-X-Leu-Leu sequence found in α/β I enhanced the inhibitory effect of this peptide and blocked estradiol-mediated transcription by about 90% (*13*). The inability of α/β III and α/β V to block estradiol-mediated transcription correlates well with their inability to bind the receptor when bound by agonist. Expression of α II, α/β III, and α/β V peptides blocked the partial agonist activity of tamoxifen (Fig. 4C). α II and α/β V were the most efficient disrupters of tamoxifen-mediated transcription, inhibiting this activity by about 90%. All peptide-Gal4 fusion proteins were expressed at similar levels, indicating that the relative differences in inhibition are not due to peptide stability (*11*). We also demonstrated that receptor stability and DNA binding are not affected by peptide expression (*11*). As expected, α/β I was unable to inhibit tamoxifen-mediated transcription. These findings are in agreement with the binding characteristics of these peptides and suggest that the pocket or pockets recognized by α II, α/β III, and α/β V are required for tamoxifen partial agonist activity. Although α/β V was shown to interact with PRB when bound by RU 486 (Fig. 3B), it was unable to block the partial agonist activity mediated by PRB/RU 486 (*11*). This result suggests that ERα/tamoxifen and PRB/RU 486 partial agonist activities are manifested differently. However, because α/β V was selected against ERα, this peptide may not bind PRB with high enough affinity to permit it to be useful as a PRB peptide antagonist.

Finally, we examined the ability of these peptides to inhibit ER transcriptional activity mediated through AP-1–responsive genes. This pathway has been proposed to account for some of the cell-specific agonist activity of tamoxifen (*14*). Both estradiol and tamoxifen activated transcription from the AP-1–responsive collagenase reporter gene, pCOL-Luc (Fig. 4D). This activity is manifest in the absence of an ERE and is believed to occur through a mechanism involving an interaction between ERα and the promoter-bound AP-1 complex (*14*). Regardless of the mechanism, each peptide was able to inhibit ERα-mediated transcriptional activity in a manner that reflected its ability to interact with the receptor in a ligand-dependent manner (Fig. 4E).

The mechanism by which tamoxifen manifests SERM activity is not yet known. Evidence presented in this study suggests that the tamoxifen-bound receptor exposes a binding site that is occupied by a coactivating protein not primarily used by the estradiol-activated receptor. The α II peptide, which interacts with both estradiol- and tamoxifen-bound receptors, inhibits the partial agonist activity of tamoxifen efficiently, while minimally affecting estradiol-mediated transcription. This result suggests that this site, although crucial for tamoxifen-mediated transcription, is dispensable for estrogen action. In addition, the ability of α/β III and α/β V to bind tamoxifen-specific surfaces and inhibit tamoxifen-mediated partial agonist activity suggests that these peptides may potentially recognize a protein contact site on ER that is critical for this activity. In this regard, we can

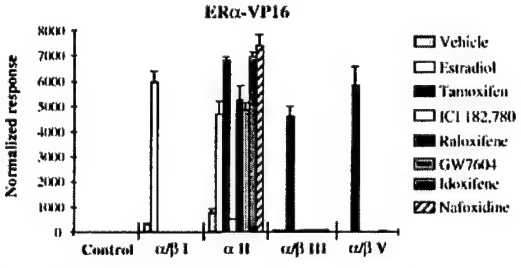

Fig. 2. ERα-peptide interactions in mammalian cells. The coding sequence of a peptide representative from each class identified was fused to the DBD of the yeast transcription factor Gal4. HepG2 cells were transiently transfected with expression vectors for ERα-VP16 and the peptide-Gal4 fusion proteins. In addition, a luciferase reporter construct under the control of five copies of a Gal4 upstream enhancer element was also transfected along with a pCMV–β-galactosidase (β-Gal) vector to normalize for transfection efficiency. Transfection of the Gal4 DBD alone is included as control. Cells were then treated with various ligands (100 nM) as indicated and assayed for luciferase and β-Gal activity. Normalized response was obtained by dividing the luciferase activity by the β-Gal activity. Transfections were performed in triplicate, and error bars represent standard error of the mean (SEM). Triplicate transfections contained 1000 ng of ERα-VP16, 1000 ng of 5× Gal4-tata-Luc, 1000 ng of peptide-Gal4 fusion construct, and 100 ng of pCMV–β-Gal (*10*).

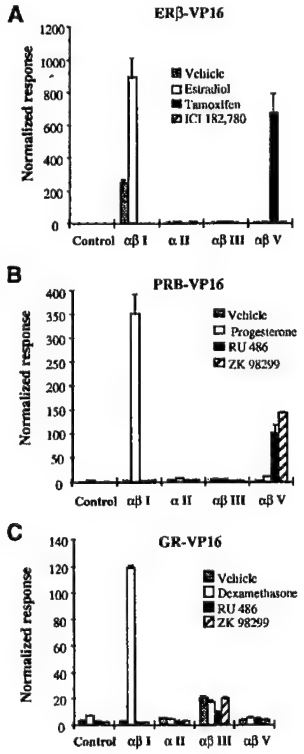

Fig. 3. Specificity of nuclear receptor–peptide interactions. Two-hybrid experiments were performed as in Fig. 2 between peptide-Gal4 fusion proteins and either (**A**) ERβ-VP16, (**B**) PRB-VP16, or (**C**) GR-VP16 (*15*). RU 486 and ZK 98299 are pan-antagonists of PRB and GR.

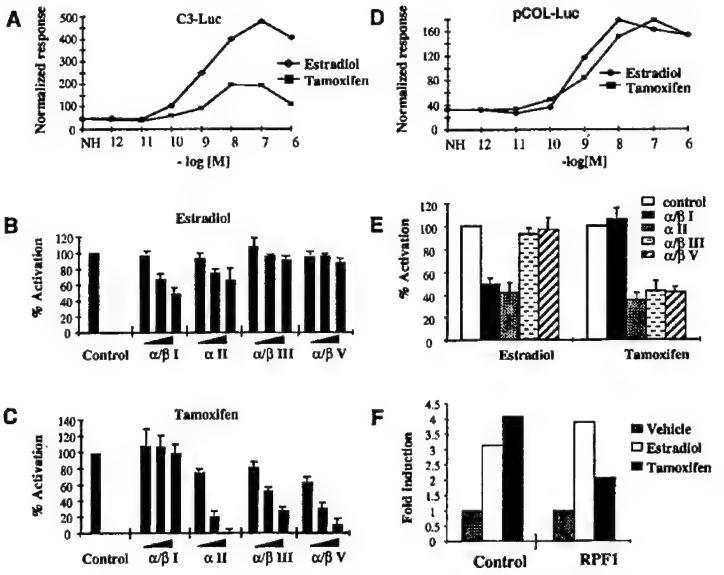

Fig. 4. Disruption of ERα-mediated transcriptional activity. **(A)** HepG2 cells were transfected with the estrogen-responsive C3-Luc reporter gene (*12*) along with expression vectors for ERα (*16*) and β-Gal and normalized as in Fig. 2. Cells were induced with either estradiol or tamoxifen as indicated and analyzed for luciferase and β-Gal activity. NH, no hormone. **(B)** HepG2 cells were transfected as in (A) except that expression vectors for peptide-Gal4 fusions were included as indicated. Control represents the transcriptional activity of estradiol (10 nM)–activated ERα in the presence of the Gal-4 DBD alone and is set at 100% activity. Increasing amounts of input plasmid for each Gal4-peptide fusion are also shown (▲) with the resulting transcriptional activity presented as percentage of activation of control. Data are averaged from three independent experiments (each performed in triplicate) with error bars representing SEM. Triplicate transfections contained 1000 ng of C3-Luc, 1000 ng of ERα expression vector, 100 ng of pCMV–β-Gal, and either 100, 500, or 1000 ng of peptide-Gal4 fusion construct. **(C)** Same as in (B) except that 4-OH tamoxifen (10 nM) was used to activate the receptor. **(D)** HepG2 cells were transfected with the AP-1–responsive collagenase reporter gene construct (pCOL-Luc) (*12*) and expression vectors for ERα and β-Gal. Cells were then induced with either estradiol or tamoxifen as indicated. **(E)** Same as (D), except that peptide-Gal4 fusion constructs were also transfected as indicated. Control represents the transcriptional activity of either estradiol- or tamoxifen (100 nM)–activated ER in the presence of the Gal4 DBD alone and is set at 100% activity. The transcriptional activity of estradiol and tamoxifen is shown in the presence of each Gal4-peptide fusion with the resulting transcriptional activity presented as percentage of activation of control. Triplicate transfections contained 1000 ng of pCOL-Luc, 1000 ng of ERα expression vector, 1000 ng of peptide-Gal4 fusion construct, and 100 ng of pCMV–β-Gal. Data are presented as in (B) and (C). **(F)** HeLa cells were transfected with the 1X-ERE-tata-Luc reporter gene along with expression vectors for ERα, β-Gal, and either RPF1 (pCDNA3-RPF1) or control vector [pcDNA3 (Invitrogen, Carlsbad, CA)]. Cells were induced with ligand (10 nM) as indicated. Data are presented as fold induction, which represents the ratio of ligand induced versus vehicle for each transfection.

demonstrate that, similar to α/β V, overexpression of RPF1 specifically represses tamoxifen-mediated partial agonist activity (Fig. 4F). However, the physiological importance of this activity remains to be determined. In summary, we have identified a series of peptide antagonists of ERα and hence validated additional target sites other than the ligand-binding pocket for drug discovery.

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6. Phage display was performed as described [L. A. Paige *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3999 (1999)]. Immulon 4 96-well plates (Dynex Technologies, Chantilly, VA) were coated with streptavidin in $NaHCO_3$ buffer (pH 8.5) at 4°C for about 18 hours. Wells were blocked with bovine serum albumin (BSA) and then washed with TBST [10 mM tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20], and 2 pmol of biotinylated vitellogenin ERE was then added per well. Plates were washed with TBST, 3 pmol of baculovirus-purified ERα or ERβ (Pan Vera, Madison, WI) was then added, and plates were incubated at room temperature for 1 hour. Hormone was then added (1 μM) along with phage library (containing about 1.5×10^9 phage) in TBST and incubated at room temperature for 1 hour. Nonbinding phage were removed by washing with TBST. Bound phage were eluted in prewarmed (50°C) 50 mM glycine-HCL (pH 2.0). Eluent was neutralized by the addition of 200 mM Na_2HPO_4 (pH 8.5), and phage were amplified in *Escherichia coli* (DH5αF′). Affinity selection was repeated three times, and individual phage were isolated from either the second or third round of amplification. Peptide sequences were then deduced by DNA sequencing.
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9. TRF assays were performed at room temperature as follows: Costar (Cambridge, MA) high-binding 384-well plates were coated with streptavidin in 0.1 M sodium bicarbonate and blocked with BSA. Twenty microliters of biotinylated ERE (100 nM in TBST) was added to each well. After a 1-hour incubation, biotin (50 μM in TBST) was added to block any remaining binding sites. The plates were washed, and 20 μl of ERα (100 nM in TBST) was added to each well. After a 1-hour incubation, the plates were washed, and 5 μl of 5 μM 4-OH tamoxifen was added to each well followed by 15 μl of solution containing the peptides conjugated to unlabeled streptavidin (prepared as described below) at a range of concentrations (from 1.67 μM in twofold dilutions). After a 30-min incubation with the 4-OH tamoxifen and conjugate, 5 μl of 400 nM europium-labeled streptavidin (Wallac, Gaithersburg, MD)–biotinylated peptide conjugate (prepared as described below) was added and incubated for 1 hour. The plates were then washed, and the europium enhancement solution was added. Fluorescent readings were obtained with a POLARstar fluorimeter (BMG Lab Technologies, Durham, NC) with a <400-nm excitation filter and a 620-nm emission filter. The streptavidin-biotinylated peptide conjugates were prepared by adding 4 pmol of biotinylated peptide per picomole of streptavidin. After incubation on ice for 30 min, the remaining biotin-binding sites were blocked with biotin before addition to the ER-coated plate.
10. HepG2 cells were maintained in modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies). Transfections were performed as described [J. D. Norris *et al.*, *J. Biol. Chem.* **270**, 22777 (1995)]. pCMV–β-Gal and 5× GAL4-tata-Luc were described previously [B. L. Wagner, J. D. Norris, T. A. Knotts, N. L. Weigel, D. P. McDonnell, *Mol. Cell. Biol.* **18**, 1369 (1998)]. Gal4 DBD-peptide fusions were created as follows: Peptide-coding sequences were excised from mBAX vector with Xho I–Xba I and subcloned into pM vector (Clontech, Palo Alto, CA) with a linker sequence to generate Sal I and Xba I sites for cloning. ERα-VP16 was generated by polymerase chain reaction (PCR) of human ERα-cDNA containing Eco RI sites flanking both 5′ and 3′ termini. The resultant PCR product was then subcloned into pVP16 (Clontech). All PCR products were sequenced to ensure the fidelity of the resultant construct. 17β-estradiol, 4-hydroxy-tamoxifen, and nafoxidine were purchased from Sigma.
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17. Supported by grants to D.P.M. from the NIH (DK48807) and to M.R.H. from the Department of Defense (DAMD17-98-1-8072). ICI 182,780 was a gift from A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK), raloxifene was a gift from E. Larson (Pfizer Pharmaceuticals, Groton, CT), idoxifene was a gift from M. Gowan (SmithKline Beecham Pharmaceuticals, King of Prussia, PA), GW 7604 was a gift from T. Willson (Glaxo Wellcome Research and Development, Research Triangle Park, NC), PRB-VP16 and GR-VP16 were gifts from D. X. Wen and J. Miner (Ligand Pharmaceuticals, San Diego, CA), and RU 486 and ZK 98299 were gifts from Ligand Pharmaceuticals and Schering-AG Pharmaceuticals (Berlin, Germany), respectively.

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Estrogen receptor (ER) modulators each induce distinct conformational changes in ER α and ER β

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ABSTRACT **Estrogen receptor (ER) modulators produce distinct tissue-specific biological effects, but within the confines of the established models of ER action it is difficult to understand why. Previous studies have suggested that there might be a relationship between ER structure and activity. Different ER modulators may induce conformational changes in the receptor that result in a specific biological activity. To investigate the possibility of modulator-specific conformational changes, we have applied affinity selection of peptides to identify binding surfaces that are exposed on the apo-ERs α and β and on each receptor complexed with estradiol or 4-OH tamoxifen. These peptides are sensitive probes of receptor conformation. We show here that ER ligands, known to produce distinct biological effects, induce distinct conformational changes in the receptors, providing a strong correlation between ER conformation and biological activity. Furthermore, the ability of some of the peptides to discriminate between different ER α and ER β ligand complexes suggests that the biological effects of ER agonists and antagonists acting through these receptors are likely to be different.**

The estrogen receptor (ER) is a member of the steroid family of nuclear receptors. Like other nuclear receptors, the ER is a ligand-dependent transcriptional activator (1). In the absence of hormone, the ER resides in the nucleus of target cells where it is associated with an inhibitory heat-shock protein complex (2). On binding ligand, the receptor is activated. This process permits the formation of stable receptor dimers and subsequent interaction with specific DNA response elements located within the regulatory region of target genes (3). The DNA-bound receptor can then either positively or negatively regulate target gene transcription. Although the precise mechanism by which the ER modulates RNA polymerase activity remains to be determined, it has been shown recently that agonist-bound ER can recruit transcriptional adaptors, proteins that permit the receptor to transmit its regulatory information to the cellular transcriptional apparatus (4–6). Conversely, when occupied by antagonists, the DNA-bound receptor actively recruits corepressors, proteins that permit the cell to distinguish between agonists and antagonists (5–7). Building on this complexity was the recent discovery of a second ER, ER β, whose mechanism of action appears to be similar to, yet distinct from, ER α (8–10).

Drugs that target the ER can exhibit a variety of effects in different target tissues. For example, tamoxifen is an ER antagonist in breast tissue (11) but an ER agonist in bone (12) and uterine (13) tissue. Raloxifene is also an ER antagonist in breast tissue; however, it exerts agonist activity in bone but not uterine tissue (14). Indeed, one of the greatest challenges in understanding the pharmacology of the ER is determining how different ER ligands produce such diverse biological effects. We have explored the possibility that various ER ligands induce distinct conformational changes in the ER. These distinct conformations may, in turn, alter the interactions of the receptor with cell- and tissue-specific coactivating or corepressing proteins or even estrogen response elements (EREs), thus leading to diverse biological effects. Using limited proteolysis, we and others have shown that the ER agonist estradiol and the ER antagonist ICI 182,780 induced distinct ER conformations (15, 16). However, the picture is much more complicated than this. There is a variety of ER ligands, selective ER modulators, which are neither pure agonists nor antagonists. These ligands, which include tamoxifen and raloxifene, produce distinct tissue-specific biological effects, yet conformational differences cannot be discerned in the protease digestion assay (15, 16). It is likely that these compounds are also eliciting distinct conformational changes that affect ER activity, but the changes are too subtle to be detected by the protease digestion assay (17, 18).

In an effort to explore the relationship between ER structure and biological activity, we have used affinity-selected peptides to probe the conformational changes that occur within the ER on binding various ligands. Our results indicate that different peptide-binding surfaces on the ER are exposed in response to binding different ligands, and that these binding surfaces are distinct from those exposed on the apo-receptor. We infer from these results that different ER–ligand complexes may be able to contact different proteins within the cell, and that the overall biological response is determined by unique combinations of protein–protein interactions that occur in a given cell and promoter context.

MATERIALS AND METHODS

Materials. ER α and β were purchased from Panvera (Madison, WI). Immulon 4 96-well plates were from Dynatech. Streptavidin, 17-β estradiol, 4-OH tamoxifen, nafoxidine, clomiphene, diethylstilbestrol, progesterone, 16-α OH estrone, and estriol were purchased from Sigma. Premarin is a product of Wyeth-Ayerst Laboratories (Marietta, PA). Raloxifene is a product of Eli Lilly. ICI 182,780 was purchased from Tocris Cookson (Ballwin, MO). Anti-M13 antisera was purchased from Amersham Pharmacia. Sequencing of single-strand M13 DNA was conducted by Sequetech (Mountain View, CA). Peptide synthesis was conducted by AnaSpec (San Jose, CA). Oligonucleotides corresponding to the vitellogenin ERE, biotin- GATCTAGGTCACAGTGACCTGCG (forward) and

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Abbreviations: ER, estrogen receptor; ERE, estrogen response element; TRF, time-resolved fluorescence; DES, diethylstilbestrol.

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biotin-GATCCGCAGGTCACTGTGACCTA (reverse), were synthesized by Genosys (The Woodlands, TX).

Phage Affinity Selection. Affinity selection of phage for the various conformations of the ER was conducted essentially as described (19). Immulon 4 96-well plates were coated with streptavidin in 0.1 M sodium bicarbonate. The plates were then incubated for 1 h with 2 pmol biotinylated vitellogenin ERE per well (20), followed by incubation for 1 h with 3 pmol (monomer) ER α or ER β per well. Affinity selections were conducted with the ER in TBST (10 mM Tris·HCl, pH 8.0/150 mM NaCl/0.05% Tween 20) or in TBST containing 1 μM 17-β estradiol or 4-OH tamoxifen.

Phage ELISA. ER α or β was immobilized on the vitellogenin ERE as described for phage affinity selection. The ER was then incubated with 100 μl TBST or TBST containing the appropriate modulator. Phage (40 μl) from a 5-h culture grown in DH5αF′ cells was added directly to the wells and incubated 30 min at room temperature. Unbound phage were then removed by five washes with TBST. Bound phage were detected by using an anti-M13 antibody coupled to horseradish peroxidase. Assays were developed with 2,2-azinobis(3-ethylbenzothiazoline)-6 sulfonic acid and hydrogen peroxide for 10 min and then stopped by the addition of 1% SDS. Absorbance was measured at 405 nm in a Molecular Devices microplate reader.

Mapping Phage Binding Sites on ER α. For mapping studies, the ER α ligand-binding domain (residues 282–595) fused to glutathione *S*-transferase (GST) (a gift from Peter Kushner) and the ER α N terminus (residues 1–184) fused to GST were used. The full length ER α or domains were immobilized directly on the surface of the Immulon 4 plate. Assays were conducted as described for phage ELISA.

Time-Resolved Fluorescence Assays. Time-resolved fluorescence (TRF) assays were performed at room temperature as follows: costar high-binding 384-well plates were coated with streptavidin in 0.1 M sodium bicarbonate and blocked with BSA. Biotinylated ERE (2 pmol) was added to each well. After a 1-h incubation, biotin was added to block any remaining binding sites. The plates were washed, and 2 pmol ER α was added to each well. Following a 1-h incubation, the plates were washed and the ER modulators were added at a range of concentrations. Following a 30-min incubation with the modulators, 2 pmol of a europium-labeled streptavidin (Wallac, Gaithersburg, MD) -biotinylated peptide conjugate (prepared as described below) was added and incubated for 1 h. The plates were then washed and the europium enhancement solution was added. Fluorescent readings were obtained with a POLARstar fluorimeter (BMG Lab Technologies) by using a <400-nm excitation filter and a 620-nm emission filter. The europium-labeled streptavidin-biotinylated peptide conjugate was prepared by adding 8 pmol biotinylated peptide to 2 pmol labeled streptavidin. After incubation on ice for 30 min, the remaining biotin-binding sites were blocked with biotin before addition to the ER-coated plate.

RESULTS AND DISCUSSION

Affinity selection of phage-displayed peptide libraries (19) was conducted on both ER α and β under conditions that were predicted to place the ER in different conformations — apo-ER, estradiol-bound ER, and 4-OH tamoxifen-bound ER. Unique sets of high-affinity peptides were identified under each condition. Most notably, affinity selection of peptides in the presence of estradiol revealed a number of sequences containing an LXXLL motif (Fig. 1*A*). This motif, which is found in nuclear receptor coactivators (Fig. 1*B*), has been shown to be necessary and sufficient for their association with nuclear receptors (21). Studies have shown that the association of the LXXLL motif with the ER is accomplished via a helical region in the ligand-binding domain of the receptor that is

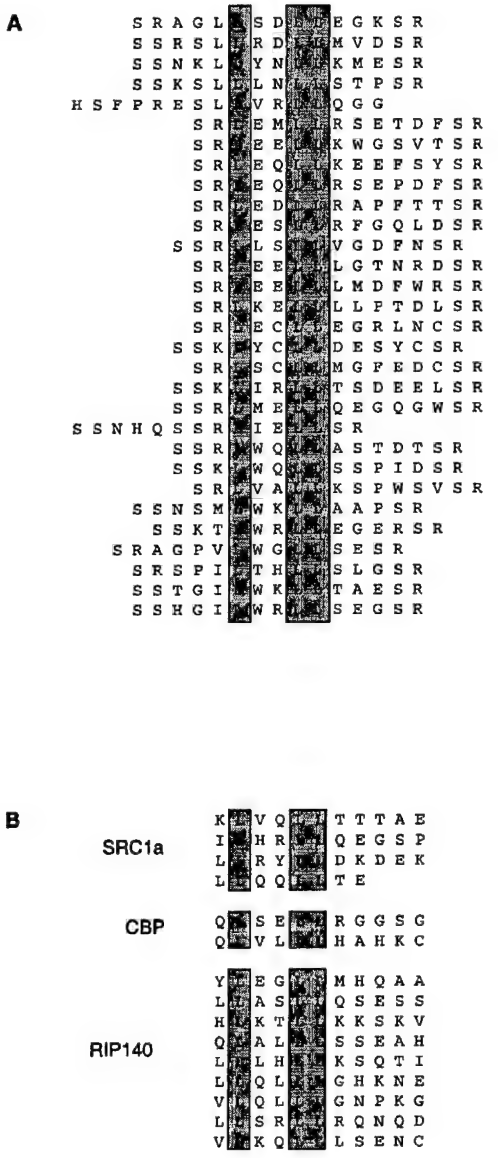

FIG. 1. (*A*) Sequences of LXXLL motif containing peptides that were affinity selected on ER α in the presence of estradiol. (*B*) Sequences of LXXLL motifs found in the nuclear receptor coactivating proteins human SRC1a (steroid receptor coactivator 1a), mouse cAMP-responsive element binding protein (CREB)-binding protein (CBP), and human RIP140.

exposed on binding estradiol. Structural studies using x-ray crystallography have shown that this region is not properly positioned in the presence of raloxifene (17) or 4-OH tamoxifen (18), thus preventing the interaction of the coactivator LXXLL motif. The identification of these sequences in the presence of estradiol indicates that the ER is undergoing conformational changes in response to ligand *in vitro* consistent with the changes that are predicted to occur *in vivo*.

All of the affinity-selected phage were evaluated by phage ELISA for binding to apo-ER α and β and to ER α and β in the presence of estradiol or 4-OH tamoxifen, as described in *Materials and Methods* and illustrated in Fig. 2. Many phage showed distinct preferential binding. Some sequences bound more strongly to the apo-receptor, while others exhibited preferential binding to the estradiol-activated or the 4-OH

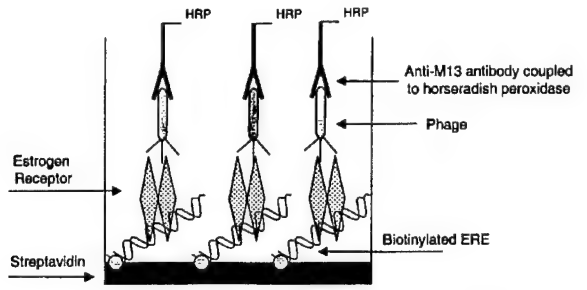

FIG. 2. Phage ELISA. A biotinylated vitellogenin ERE was immobilized on 96-well plates precoated with streptavidin. The ER was then immobilized on the ERE and incubated for 5 min in the presence of modulator before the addition of phage. Assays were conducted as described in *Materials and Methods*. HRP, horseradish peroxidase.

tamoxifen-activated receptor. No signal was observed when the assays were carried out in the absence of ER, indicating that the peptides were binding to the ER ligand complex. Based on this analysis, 11 phage expressing different peptide sequences and showing distinct binding preferences were chosen for further use as conformational probes (Fig. 3). Five of the probes have affinity for both ER α and ER β and were designated α/β I–V. Three probes were specific for ER α, designated α I–III, and three were specific for ER β, designated β I–III. The identification of distinct classes of peptides, some of which recognized both ER α and ER β and others that were receptor specific, is consistent with the primary structures of the two receptors being similar yet distinct.

The binding sites of the eight probes, α/β I–V and α I–III, were mapped on ER α by using isolated ER α ligand-binding domain, an amino terminal domain, and the full length ER. Assays were conducted by using the format shown in Fig. 2, except that the domains and the full length receptor were directly immobilized on the plastic surface of the well. All of the probes, except α I, bound to the ligand-binding domain. The α I probe, which binds only to the full length protein, may be binding to a site that is created by the tertiary structure formed by the interaction between receptor domains (data not shown). Whereas the binding of the probes requires the presence of the ER ligand-binding domain, we cannot at this time formally exclude the possibility that the binding surface for some probes is created by the combination of ligand and receptor. However, crystal structures of the ER ligand-binding domain complexed with ligand indicate that helix 12 is positioned over the ligand-binding site such that the probes may be sterically hindered from binding directly to the ligand (17, 18).

Next, we evaluated the binding of each of the probes to ER α and ER β in the presence of a variety of ER ligands that have distinct biological activities (22–31). The goal was to determine

Probe	Method of Isolation (receptor and condition)	
α/β I	ER α	estradiol
α/β II	ER α	buffer
α/β III	ER α	4-OH tamoxifen
α/β IV	ER β	4-OH tamoxifen
α/β V	ER β	4-OH tamoxifen
α I	ER α	buffer or estradiol
α II	ER α	estradiol or 4-OH tamoxifen
α III	ER α	buffer
β I	ER β	4-OH tamoxifen
β II	ER β	estradiol
β III	ER β	buffer

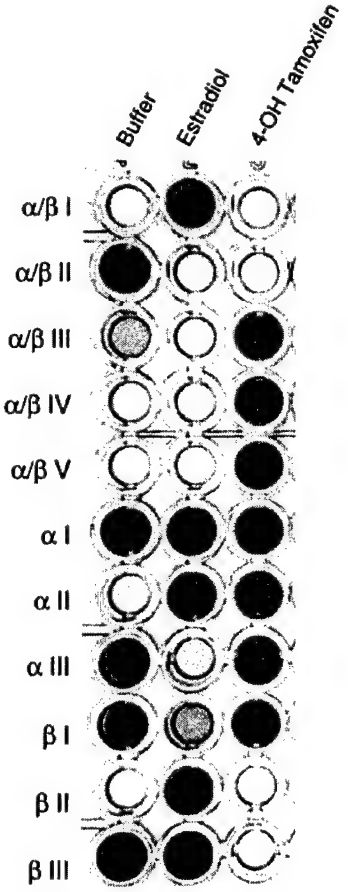

FIG. 3. Analysis of the binding specificity of the conformational probes was conducted by phage ELISA as described in *Materials and Methods*. Estradiol and 4-OH tamoxifen concentrations were 1 μM. The probes α/β I-α/β V are shown only for ER α. The binding patterns of these probes on ER β were similar. Sequences of the probes are as follows: α/β I, SSNHQSSRLIELLSR; α/β II, SAPRATISHYLMGG; α/β III, SSWDMHQFFWEGVSR; α/β IV, SRLPPSVFSMCGSEVCLSR; α/β V, SSPGSREWFKDMLSR; α I, SSEYCFYWDSAHCSR; α II, SSLTSRDFGSWYASR; α III, SRTWESPLGTWEWSR; β I, SREWEDGFGGRWLSR; β II, SSLDLSQFPMTASFLRESR; β III, SSEACVGRWMLCEQLGVSR.

whether each of the ligands would induce a conformational change in the ER that would alter the binding pattern of the probes, thus producing a "fingerprint" for each compound. The ligands used for this study include the ER agonists estradiol, estriol, and diethylstilbestrol (DES); the selective ER modulators 4-OH tamoxifen, nafoxidine, clomiphene, and raloxifene; the antagonist ICI 182,780; and the estradiol metabolite 16-α-OH estrone. Premarin, the mixture of conjugated estrogens used as estrogen replacement therapy, was also included, but it should be noted that many of the components of Premarin must be metabolically activated. Thus, their action may not be detected in this *in vitro* assay. Buffer only (apo-receptor) and progesterone were included as controls. As shown in Fig. 4, each of the ligands tested did indeed alter the binding pattern of the probes, producing a distinct fingerprint for each, whereas the pattern produced by progesterone was indistinguishable from that produced by buffer.

The unique ligand-dependent binding patterns of the probes indicates that each ligand induces a receptor conformational change that differentially exposes peptide-binding surfaces. The binding patterns for estradiol and ICI 182,780 are distinct on both ER α and β, confirming the conformational change illustrated by the earlier protease digestion studies (15). The protease digestion assay, which relies on the location of cleavage sites for detection of conformational changes, could distinguish between conformational changes induced by estradiol and 4-OH tamoxifen or estradiol and ICI 182,780. How-

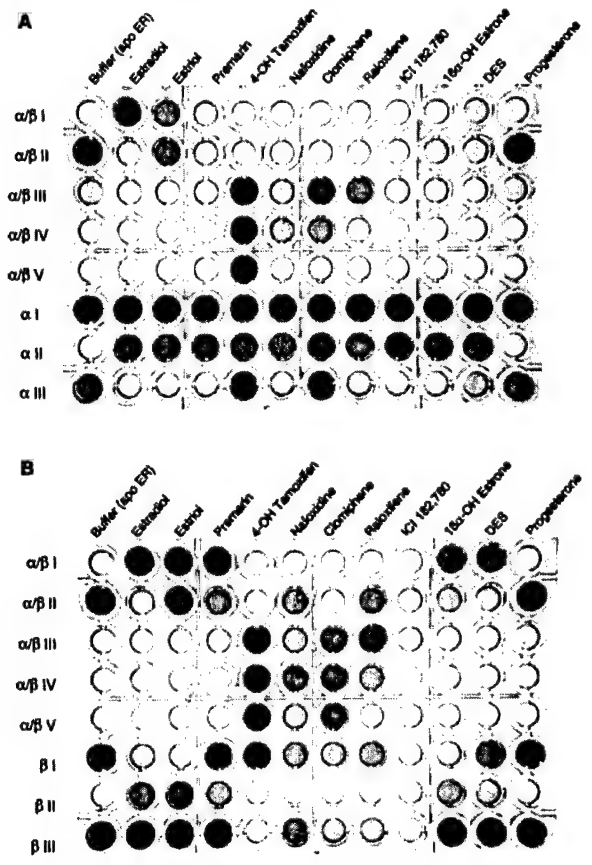

FIG. 4. Fingerprint analysis of ER modulators on (*A*) ER α and (*B*) ER β. Immobilized ER was incubated with estradiol (1 μM), estriol (1 μM), Premarin (10 μM), 4-OH tamoxifen (1 μM), nafoxidine (10 μM), clomiphene (10 μM), raloxifene (1 μM), ICI 182,780 (1 μM), 16α-OH estrone (10 μM), DES (1 μM), or progesterone (1 μM). Phage ELISAs were conducted as described in *Materials and Methods*.

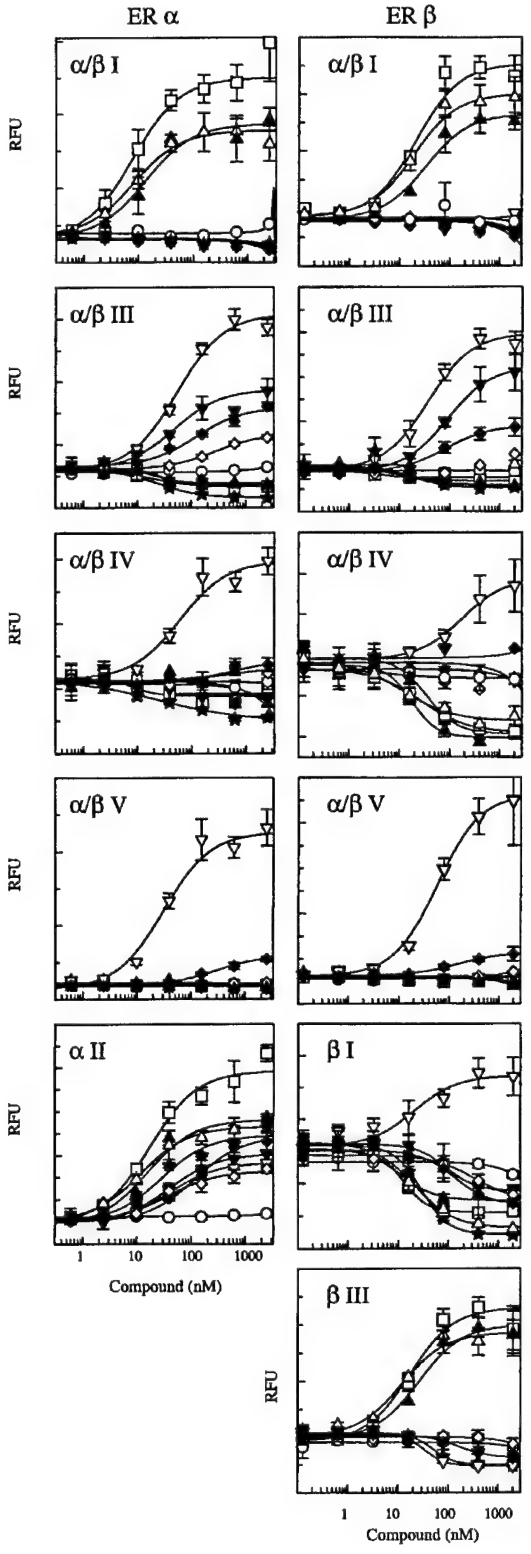

FIG. 5. Comparison of the binding of the peptide probes to ER α or ER β in the presence of modulators using TRF. Assays were conducted as described in *Materials and Methods*. (○), buffer, apo-receptor; (□) 17β estradiol;(△) estriol; (▲) DES; (▽) 4-OH tamoxifen; (▼) raloxifene; (◇)nafoxidine; (◆) clomiphene; (⋆) ICI 182,780. RFU, relative fluorescence units.

Table 1. Binding of the peptide probe to ER α and ER β in the presence of modulators

	EC_{50} for ER peptide probe										
	ER α					ER β					
Modulator	α/β I	α/β III	α/β IV	α/β V	α II	α/β I	α/β III	α/β IV	α/β V	β I	β III
Buffer											
17β-Estradiol	8	18	8		17	22	6	27		13	17
Estriol	8	19	45		12	20	16	12		21	12
4-OH tamoxifen		55	60	31	42		37	180	50	21	34
Nafoxidine		290	370		39		230			320	
Clomiphene		140	710	280	120		82		150	140	120
Raloxifene		49			42		90			90	160
ICI 182, 780		26	25		29		18	35		29	48
Diethylstilbesterol	13	30			16	34	15	18		11	25

EC_{50} is defined as the concentration, in nanomolar, of a given modulator required to achieve a 50% change in the binding of the probe to the receptor. The change in conformation may result in an increase or decrease in the affinity of the probe for the receptor.

ever, it was unable to distinguish between changes induced by 4-OH tamoxifen and other ER modulators such as ICI 182,780 (15). The fingerprint assay, however, clearly indicates that unique peptide-binding surfaces are exposed on both ER α and β in the presence of 4-OH tamoxifen that are not exposed in the presence of ICI 182,780. Tamoxifen, nafoxidine, and clomiphene contain the same triphenylethylene core structure. These three compounds, although similar in structure, produce distinct biological effects. Therefore, it might be predicted that these compounds would induce similar, yet distinct, conformational changes in the receptors. The fingerprint assay shows that the probes α/β III, IV and V, which have high affinity for the ER in the presence of 4-OH tamoxifen, have lower affinity for the ER complexed with nafoxidine and clomiphene, indicating that the exposure of these peptide-binding surfaces differs in the presence of these compounds. The α III probe more clearly differentiates these three compounds. The fingerprint assay also differentiates 4-OH tamoxifen and raloxifene. The probes α/β III, IV, and V have reduced affinity for both ER α and β in the presence of raloxifene compared with 4-OH tamoxifen. The probes α/β II, β I, and β III further distinguish ER β conformational changes induced by these two compounds. The fingerprint pattern produced by Premarin is distinct compared with other agonists; however, Premarin's activities are caused by a mixture of components. It would be interesting to assess the binding patterns of the probes in the presence of each of the purified activated components of Premarin.

To confirm that the binding of the probes to the ER depended on the peptide expressed on the surface of the phage, biotinylated peptides corresponding to the phage sequences were synthesized with biotin attached to a C-terminal lysine. The peptides were coupled to europium-labeled streptavidin and binding studies were conducted by using TRF spectroscopy, as described in *Materials and Methods*. The concentrations of the modulators were varied from picomolar to micromolar, and the binding of these probes to the ER was measured. The results, shown in Fig. 5, indicate that the peptides are indeed conferring the binding specificity. Comparison of the fluorescence values obtained from the TRF binding assays and the signals obtained in the phage ELISA fingerprint indicate that the two methods produce similar patterns. However, the binding assay also provides an indication of the potency of each compound to induce the conformational change required for peptide binding (Table 1). Taken together, these results indicate that conversion of the fingerprint assay from phage to peptides will provide an even more sensitive assay for detecting conformational change.

One of the most notable observations from the TRF binding assays is that the binding of the β I probe to ER β is enhanced in the presence of the selective ER modulator 4-OH tamoxifen and reduced in the presence of other SERMs such as raloxifene, nafoxidine, and clomiphene. The reduction in binding observed with these compounds is similar to the reduction observed with agonists such as estradiol, estriol, and DES.

We have identified peptides that serve as conformational probes of the ER α and β. Many probes bind to both receptors, while other probes bind to either the α or β receptor. Consistent with the two receptors having regions of high homology and other more divergent regions, these results indicate that the receptors have some binding surfaces in common, while others are unique. The implications of this are that both receptors may contact some of the same regulatory proteins in the cell, yet there may be additional proteins that specifically regulate either ER α or β action.

We have used our peptidic probes to show that both receptors undergo distinct conformational changes as a result of binding different ligands. The probes not only reveal receptor conformational changes by their relative changes in affinity, but they also identify unique binding surfaces on the two receptors. These binding surfaces may, in fact, be the surfaces that interact with various coregulatory proteins in response to different ligands. For example, many peptides selected with the estradiol-activated receptor contained sequences found in nuclear receptor coactivators, as illustrated by the peptides containing the LXXLL motif (Fig. 1). These peptide probes are probably mimicking the interaction between the receptor and coactivating proteins. Potentially, these probes can be used to identify heretofore unknown receptor–protein interactions.

Additional applications of the probes lie in the area of detection of ER modulators. One or more probes can be used to set up a high-throughput screen to identify modulators of ER activity. We anticipate that compounds that bind to the ER will alter receptor conformation and hence alter the binding patterns of the probes. The sites targeted by the screen may not be *bona fide* protein–protein interaction surfaces, but may represent sites exposed in the presence of a specific ligand and thus serve as markers for specific conformations. The fingerprinting technique may also be applied to quickly classify hits from a screen into different categories such as agonist (resembling the estrogen pattern), antagonist (resembling the ICI 182,780 pattern), mixed (resembling the tamoxifen pattern), or novel effectors, before assessing them in a cell-based assay. Fingerprinting may also be used to determine structure activity relationships and to rapidly assess compounds after chemical modification during lead optimization.

This is, to our knowledge, the first technique described that can distinguish between ER conformations induced by ligands both between and within ligand classes. The data gathered with this assay provide strong evidence that the biological activity of the ER can be linked to the conformation induced on binding ligand. A strength of this fingerprinting technique is that it is broadly applicable to any protein or receptor that

undergoes structural changes on binding of a ligand or substrate. We are currently applying this technique to additional receptors and signaling proteins to aid in assessing conformational changes in response to chemical modulators of activity.

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Meeting Abstract #1:

Identification and characterization of proteins which interact with ER-AF2 in a manner distinct from the p160 class of coactivators.

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The transcriptional activity of human estrogen receptor-alpha (ERα) is manifest in a cell-selective manner utilizing its two activation functions, AF-1 and AF-2. Since interaction with coactivators is essential for ERα to function as a transcriptional activator, it is hypothesized that cell-type specific coactivators may exist and contribute to the observed cell-selective transcriptional activity of different ERα ligands. Most of the coactivators identified to date contain an LXXLL sequence, a motif with which coactivators use to interact with the ligand binding domain (LBD) of the nuclear receptors. Mutations within ER-AF2:D538E542D545 to N538Q542N545 (ER-3x) have been shown to abolish the ability of p160-type coactivators such as SRC-1 and GRIP-1 to interact with ER through their LXXLL motifs. As anticipated, these mutations also destroy ER transcriptional activity in most cell contexts. In HepG2 cells, however, the transcriptional activity of the ER-3x mutant is similar to that of the wild type receptor. We believe that these results can be explained in either of two ways: 1) in HepG2 cells, AF-1 is dominant and AF-2 is not required, or 2) in this cell line a cofactor exists whose interaction with ER does not require an intact AF-2. Using a combinatorial approach to screen a library of different LXXLL motifs, we discovered three functionally distinct LXXLL sequences, of which one, the class III peptides, interacts equally well with ER-wt and ER-3x. More importantly, when peptides containing either the class III or GRIP-1 LXXLL-motifs were over-expressed in HepG2 cells, they efficiently inhibited wild type ER activity; however, under the same conditions only the class III peptide was able to inhibit ER-3x transcriptional activity. These data suggest that in HepG2 cells, a cofactor which utilizes a class III type LXXLL motif may exist and may be important for ER function. Based on this observation, we initiated a yeast two-hybrid screen using a mutant ER LBD which contains the 3x mutations as a bait to search for novel coactivators that have the ability to interact with ER and modulate its transcriptional activity. We have now identified several clones that interacted with the mutant ER-LBD in a ligand-dependent manner in yeast. Detailed characterization and functional analysis of these clones are in progress. [Supported by DK48807 and DAMD17-99-1-9173]

Meeting Abstract #2:

Probing the molecular determinants of receptor-cofactor binding specificity using combinatorial libraries of receptor interacting peptides.

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One of the distal steps in transcriptional activation by estrogen receptor (ER) is the recruitment by ligand-bound receptor of one of a number of coactivator proteins. This activity permits ER to interact with the general transcription machinery and exert its regulatory actions on target gene promoters. It has now emerged that one effect of agonist binding is to induce a conformational change within ER, permitting the interaction of ER helices 3 and 12, and the subsequent formation of a pocket which allows the coactivator proteins to dock. These observations suggest that receptor antagonists inhibit ER transcriptional activity by affecting the formation of the coactivator binding pocket and reducing the affinity of ER for coactivators. Although an ER-specific coactivator protein remains to be identified, several coactivators have been identified which potentiate the transcriptional activity of ER and other members of the steroid receptor superfamily. Furthermore, the finding that these coactivators use a highly conserved LXXLL motif to interact with the receptors made it uncertain as to whether receptor-cofactor interactions were determined by simple competition or if there was some specificity built into the system. In order to address these possibilities, we undertook a molecular approach to dissect the LXXLL-ER interaction and to evaluate the role of flanking sequences in influencing these interactions. To approach this problem we utilized phage display technology to screen 10×10^7 variations of the core LXXLL motif. Using estradiol-activated ER as a target we identified a number of phage which encoded high affinity ER-interacting peptides. Using the sequence information derived from these phage, we constructed a series of GAL4-peptide fusions and assessed their ability to interact with ERα, ERβ, GR and PR using a two-hybrid assay in mammalian cells. The results of this assay confirmed that the LXXLL motif was permissive for nuclear receptor binding but it also revealed that sequences flanking this motif were important determinants of specificity. Thus, as expected, not all LXXLL motifs are the same. This suggests that within a cell, specificity and not just mass action influences the ability of a nuclear receptor to find a required cofactor. In an effort to understand the mechanism underlying this observed specificity, we assayed the ability of these peptide fusions to interact with a series of ER helix-12 mutants. Using this approach we noticed that mutation of the conserved hydrophobic residues in this helix abolished ER-AF-2 function and blocked the interaction of all LXXLL peptides with ER. Disruption of helix 12 by mutating the three conserved charged residues (D538N/E542Q/D545N) prevented most peptides from binding and also abolished AF-2 function. However, a large number of the LXXLL-containing peptides studied were unaffected by this manipulation. This is an important observation since the latter mutation also blocks the interaction of ER with GRIP-1 and SRC-1. Cumulatively, our data indicate that the steroid receptors display distinct preferences for different classes of LXXLL motifs, suggesting a molecular basis for cofactor-receptor specificity. Importantly, however, they also indicate that AF-2 function and coactivator binding are not synonymous, a result which indicates that there are likely to be additional cofactors distinct from SRC-1 and GRIP-1 which remain to be discovered.